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(71) Applicant: LEXINGEN PHARMACEUTICALS CORP. [US/US]; 125 Hartwell Avenue, Lexington, MA 02173 (US).

(72) Inventors: LO, Kin-Ming; 6 Carol Lane, Lexington, MA 02420 (US). LI, Yue; 53 Loomis Street, Bedford, MA 01730 (US). GILLIES, Stephen, D.; 159 Sunset Road, Carlisle, MA 01741 (US).

(74) Agent: BRESNAHAN, Maureen, B.; Testa, Hurwitz & Thibeault, LLP, High Street Tower, 125 High Street, Boston, MA 02110 (US).

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(54) Title: EXPRESSION AND EXPORT OF ANGIOSTATIN AND ENDOSTATIN AS IMMUNOFUSIS

(57) Abstract

Disclosed are nucleotide sequences, for example, DNA or RNA sequences, which encode an immunoglobulin Fc–angiogenesis inhibitor fusion protein. The angiogenesis inhibitors can be angiostatin, endostatin, a plasminogen fragment having angiostatin activity, or a collagen XVIII fragment having endostatin activity. The nucleotide sequences can be inserted into a suitable expression vector and expressed in mammalian cells. Also disclosed is a family of immunoglobulin Fc–angiogenesis inhibitor fusion proteins that can be produced by expression of such nucleotide sequences. Also disclosed are methods using such nucleotide sequences and fusion proteins for treating conditions mediated by angiogenesis.

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EXPRESSION AND EXPORT OF ANGIOGENESIS INHIBITORS AS IMMUNOFUSINS

Field of the Invention

This invention relates generally to methods and compositions for making and using fusion proteins containing an angiogenesis inhibitor. More particularly, the invention relates to methods and compositions for making and using fusion proteins called immunofusins which contain an immunoglobulin Fc region and an angiogenesis inhibitor.

Background of the Invention

Two potent angiogenesis inhibitors, angiostatin (O'Reilly et al. (1994) Cell 79:315) and endostatin (O'Reilly et al. (1997) Cell 88:277), were discovered and found to be generated naturally by primary tumors. Both proteins are specific inhibitors of endothelial cell proliferation and inhibit tumor growth by blocking angiogenesis, the formation of new blood vessels that nourish tumors. Studies have shown that these angiogenesis inhibitors are non-toxic even at very high doses and that they may suppressed the growth of metastases and primary tumors may regress to a dormant microscopic state. Both inhibitors were identified as proteolytic fragments of much larger intact molecules. Angiostatin was found to be a fragment of plasminogen, and endostatin a fragment of collagen XVIII.

These two proteins have generated great interest in the cancer area because they have been shown to suppress the growth of many different types of tumors in mice, with no obvious side effects or drug resistance. Traditional chemotherapy generally leads to acquired drug resistance caused primarily by the genetic instability of cancer cells. Rather than targeting cancer cells, therapies using angiogenesis inhibitors target the normal endothelial cells, which support the growth of the tumor. Because endothelial cells are genetically stable, it is possible that angiogenesis inhibitor therapies may result in less drug resistance. Studies indicate that drug resistance did not develop in mice exposed to prolonged anti-angiogenic therapy using endostatin. Furthermore, repeated cycles of endostatin treatment in mice resulted in prolonged

tumor dormancy and no recurrence of tumors following discontinuation of therapy (Boehm et al. (1997) Nature 390:404).

Despite promising results in mice, it has not been possible to produce clinical grade soluble, active angiostatin and endostatin in commercial quantities using *E. coli*, baculoviral, yeast, and mammalian expression systems. Expression in *E. coli* yielded insoluble protein aggregates of undefined composition, which could not be injected into humans. Other production methods, such as baculovirus and mammalian expression systems, yielded very low levels of the recombinant proteins (O'Reilly et al. (1997) Cell 88:277).

The poor yields of the expression systems to date may be explained by both angiostatin and endostatin being internal fragments of much larger proteins. The truncated proteins may not fold properly in the absence of the residues that are cleaved from the precursor molecules. For example, angiostatin has 26 cysteine residues which form numerous disulfide bonds. Expression of angiostatin by itself may not provide the optimal environment for these numerous disulfide bonds to form correctly in the secretory pathway. Also, the recombinant endostatin protein produced in *E. coli* precipitated during dialysis, possibly due to the hydrophobicity of endostatin (O'Reilly et al. (1997) Cell 88:277).

A major hurdle with the use of angiostatin and endostatin in their present forms is that relatively large amounts of proteins have to be injected daily for weeks to months to achieve the desired clinical outcome. For example, in current mouse models, dosages of 20 mg/kg/day of endostatin are needed to demonstrate optimal efficacy (Boehm et al. (1997) Nature 390:404). Given that there is an urgent need to test endostatin and angiostatin clinically, a production method that can generate large quantities of clinical grade material is important.

One expression system that has been used to produce high level expression of fusion proteins in mammalian cells is a DNA construct encoding, a signal sequence, an immunoglobulin Fc region and a target protein. The fusion product of this construct generally is termed an "immunofusin." Several target proteins have been expressed successfully as immunofusins which include: IL2, CD26, Tat, Rev, OSF-2, βIG-H3, IgE Receptor, PSMA, and gp120. These expression constructs are disclosed in U.S. Patent No. 5,541,087 and U.S. Patent No. 5,726,044, the disclosures of which are incorporated herein by reference.

A major purpose of expressing recombinant fusion proteins in mammalian cells has been to attempt to confer novel or useful properties to the hybrid molecules, e.g., proper folding, increased solubility, targeting of a cytokine or toxin *in vivo*, Fc receptor binding, complement fixation, protein A binding, increased circulation half-life, and increased ability to cross the blood-brain barrier. Examples of recombinant fusion proteins produced in mammalian cells include cytokine immunoconjugates (Gillies et al. (1992) Proc. Natl. Acad. Sci. USA 89:1428; Gillies et al. (1993) Bioconjugate Chemistry 4:230), immunoadhesins (Capon et al. (1989) Nature 337:525), immunotoxins (Chaudhary et al. (1989) Nature 339:394), and a nerve growth factor conjugate (Friden et al. (1993) Science 259:373). Each of the foregoing publications is incorporated herein by reference.

It is an object of the invention to provide novel DNAs which facilitate efficient production and secretion of angiogenesis inhibitors in a variety of mammalian host cells. It is another object of the invention to provide methods for treating mammals with nucleic acids encoding, or amino acid sequences defining angiogenesis inhibitor proteins, including non-native, biosynthetic, or otherwise artificial proteins such as proteins which have been created by rational design.

Summary of the Invention

The present invention features methods and compositions useful in making and using fusion proteins containing an angiogenesis inhibitor protein. The fusion proteins can facilitate a high level expression of biologically active angiogenesis inhibitor proteins. The angiogenesis inhibitor proteins can then be cleaved from the fusion protein and combined with a pharmaceutically acceptable carrier prior to administration to a mammal, for example, a human. Alternatively, nucleic sequences encoding, or amino acid sequences defining the angiogenesis inhibitor containing fusion proteins can be combined with a pharmaceutically acceptable carrier and administered to the mammal.

In one aspect, the invention provides nucleic acid molecules, for example, DNA or RNA molecules, encoding a fusion protein of the invention. The nucleic acid molecule encodes a signal sequence, an immunoglobulin Fc region, and at least one target protein, also referred to herein as the angiogenesis inhibitor protein, selected from the group consisting of angiostatin, endostatin, a plasminogen fragment having angiostatin activity, a collagen XVIII fragment

having endostatin activity, and combinations thereof. In a preferred embodiment, the nucleic acid molecule encodes, serially in a 5' to 3' direction, the signal sequence, the immunoglobulin Fc region and the target protein sequence. In another preferred embodiment, the nucleic acid molecule encodes, serially in a 5' to 3' direction, the signal sequence, the target sequence, and immunoglobulin Fc region.

In another preferred embodiment, the immunoglobulin Fc region comprises an immunoglobulin hinge region and preferably comprises at least one immunoglobulin constant heavy region, for example, an immunoglobulin constant heavy 2 (CH_2) domain, an immunoglobulin constant heavy 3 (CH_3) domain), and depending upon the type of immunoglobulin used to generate the Fc region, optionally an immunoglobulin constant heavy region 4 (CH4) domain. In a more preferred embodiment, the immunoglobulin Fc region comprises a hinge region, a CH_2 domain and a CH_3 domain. Under certain circumstances, the immunoglobulin Fc region preferably lacks at least the CH_1 domain. Although the immunoglobulin Fc regions may be based on any immunoglobulin class, for example, IgA, IgD, IgE, IgG, and IgM, immunoglobulin Fc regions based on IgG are preferred.

In another embodiment, the nucleic acid of the invention can be incorporated in operative association into a replicable expression vector which can then be transfected into a mammalian host cell. In another preferred embodiment, the invention provides host cells harboring such nucleic acid sequences of the invention.

In another aspect, the invention provides a fusion protein comprising an immunoglobulin Fc region linked, either directly through a polypeptide bond or by means of a polypeptide linker, to a target protein selected from the group consisting of angiostatin, endostatin, a plasminogen fragment having angiostatin activity, a collagen XVIII fragment having endostatin activity, and combinations thereof. The target protein may be fused via its C-terminal end to an N-terminal end of the immunoglobulin Fc region. However, in a more preferred embodiment the target protein is fused via its N-terminal end to a C-terminal end of the immunoglobulin Fc region.

In another embodiment, the fusion protein may comprise a second target protein selected from the group consisting of angiostatin, endostatin, a plasminogen fragment having angiostatin activity, and a collagen XVIII fragment having endostatin activity. In this type of construct the

first and second target proteins can be the same or different proteins. For example, in a preferred embodiment, the fusion protein comprises a first target protein of angiostatin, an immunoglobulin Fc region and a second target protein of endostatin. The first and second target proteins may be linked together, either directly or by means of a polypeptide linker. Alternatively, both target proteins may be linked, either directly or via a polypeptide linker, to the immunoglobulin Fc region. In the latter case, the first target protein is connected to an N-terminal end of the immunoglobulin Fc region and the second target protein is connected to a C-terminal end of the immunoglobulin Fc region.

In another embodiment, two fusion proteins may associate, either covalently, for example, by a disulfide or peptide bond, or non-covalently, to produce a multimeric protein. In a preferred embodiment, two fusion proteins are associated covalently by means of one or more disulfide bonds through cysteine residues, preferably located within immunoglobulin hinge regions disposed within the immunoglobulin Fc regions of both chains.

In a preferred embodiment, the target protein comprises a plasminogen fragment having a molecular weight of approximately 40 kD and, optionally comprises, an amino acid sequence as set forth in SEQ ID NO: 3. In another preferred embodiment, the target protein comprises a collagen XVIII fragment having an amino acid sequence set forth in SEQ ID NO: 1. Furthermore, the target protein can be full-length angiostatin or endostatin or bioactive fragments thereof. The source of the target protein in generating certain fusion proteins will depend upon the intended use of the target protein. For example, if the target protein is to be administered to a human, the target protein preferably is of human origin.

In another aspect, the invention provides methods of producing a fusion protein comprising an immunoglobulin Fc region and a target protein selected from the group consisting of angiostatin, endostatin, a plasminogen fragment having angiostatin activity, and a collagen XVIII fragment having endostatin activity. The method comprises the steps of (a) providing a mammalian cell containing a DNA molecule encoding such a fusion protein, either with or without a signal sequence, and (b) culturing the mammalian cell to produce the fusion protein. The resulting fusion protein can then be harvested, refolded, if necessary, and purified using conventional purification techniques well known and used in the art. Assuming that the fusion protein comprises a proteolytic cleavage site disposed between the immunoglobulin Fc region

and the target protein, the target can be cleaved from the fusion protein using conventional proteolytic enzymes and if necessary, purified prior to use.

In another aspect, the invention provides methods for treating mammals, for example, a human, in need of an angiogenesis inhibitor based therapy. For example, it is contemplated that the angiogenesis inhibitors of the invention may be administered to a human afflicted with a tumor. Treatment with the angiogenesis inhibitor may slow down or stop tumor growth and, under certain circumstances, may cause tumor regression. Treatment may include administering to the mammal an amount of the angiogenesis inhibitor in an amount sufficient to slow down or stop tumor growth. The angiogenesis inhibitor may be provided in the form of a fusion protein or as a nucleic acid, preferably operatively associated with an expression vector, in combination with a pharmaceutically acceptable carrier.

The foregoing and other objects, features and advantages of the present invention will be made more apparent from the detailed description, drawings, and claims that follow.

Brief Description of the Drawings

Figures 1A-1F are schematic illustrations of exemplary angiogenesis inhibitor fusion proteins constructed in accordance with the invention (see Examples 10-15). The Figures depict, respectively, Figure 1A, Fc-Kringle 1 of Angiostatin; Figure 1B, Fc-inner Kringle 1 of Angiostatin; Figure 1C, Fc-Endostatin-GlySer linker-inner Kringle 1 of Angiostatin; Figure 1D, Fc-Endostatin-GlySer linker-Kringle 1 of Angiostatin; Figure 1E, Fc-Endostatin-GlySer linker-Angiostatin; Figure 1F, Angiostatin-Fc-Endostatin. The vertical lines represent optional disulfide bonds connecting cysteine residues (C) disposal within a hinge region of the Fc molecule.

Detailed Description of the Invention

The invention provides fusion proteins, referred to herein as immunofusins, which were useful in the production of commercial quantities of clinical grade angiogenesis inhibitors. The angiogenesis inhibitors may be cleaved from the immunofusin protein constructs prior to use. However, it is contemplated that the immunofusins or nucleic acids encoding the immunofusins may be administered directly to mammals in need of treatment with an angiogenesis inhibitor.

The invention thus provides fusion proteins comprising an immunoglobulin Fc region and at least one target protein, referred to herein as an angiogenesis inhibitor. The angiogenesis inhibitor preferably is selected from the group consisting of angiostatin, endostatin, a plasminogen fragment angiostatin activity, a collagen XVIII fragment having endostatin activity. It is contemplated, however, that other polypeptides having angiogenesis inhibitor activity, now known or late discovered, may be expressed as fusion proteins of the type described herein.

Six exemplary embodiments of protein constructs embodying the invention are illustrated in the drawing as Figures 1A-1F. Because dimeric constructs are preferred, all are illustrated as dimers cross-linked by a pair of disulfide bonds between cysteines on adjacent subunits. In the drawings, the disulfide bridges are depicted as linking together the portions of two immunoglobulin Fc regions via an immunoglobulin hinge region, and thus are characteristic of native forms of these molecules. While constructs including the hinge region of Fc are preferred and have been shown promise as therapeutic agents, the invention contemplates that the crosslinking at other positions may be chosen as desired. Furthermore, under some circumstances, dimers or multimers useful in the practice of the invention may be produced by non-covalent association, for example, by hydrophobic interaction.

Because homodimeric constructs are important embodiments of the invention, Figure 1 illustrates such constructs. It should be appreciated that heterodimeric structures also are useful but, as is known to those skilled in the art, often can be difficult to purify. However, viable constructs useful to inhibit angiogenesis in various mammalian species, including humans, can be constructed comprising a mixture of homodimers and heterodimers. For example, one chain of the heterodimeric structure may comprise endostatin and the another may comprise angiostatin.

Figure 1A illustrates a dimer construct produced in accordance with the procedure set forth in Example 10. Each monomer of the dimer comprises an immunoglobulin Fc region 1 including a hinge region, a CH_2 domain and a CH_3 domain. Attached directly to the C terminus of the Fc region 1 is the first Kringle region of angiostatin 2, both inner and outer rings. Figure 1B shows a second embodiment of the invention (see Example 11) comprising the same Fc region as in Figure 1A, this time having only the inner ring of Kringle one of angiostatin 3 attached to the C terminal end of the Fc region 1. Figures 1C through 1E depict various embodiments of the protein constructs of the invention, which include as a target protein plural angiogenesis inhibitors arranged in tandem and connected by a linker. In Figure 1C, the target protein comprises full-length endostatin 4, a polypeptide linker 5, and the inner ring of Kringle one of angiostatin 3. Figure 1D depicts a protein comprising an Fc region the same as that of Figure 1A and a target protein comprising a full-length endostatin 4, a polypeptide linker 5, and a full Kringle one region of angiostatin (both inner and outer rings) 2. Figure 1E differs from the construct of Figure 1D in that the most C terminal protein domain comprises a full-length copy of angiostatin 7.

Although Figures 1A-1E represent Fc-X type constructs, where X is the target protein, it is contemplated that X-Fc type constructs may also be useful in the practice of the invention. Furthermore, it is contemplated the useful proteins of the invention may also be depicted by the formula X-Fc-X, wherein the Xs may represent the same or different target proteins. Figure 1F depicts such a construct which comprises in an N- to C-terminal direction, full-length human angiostatin 7, a human immunoglobulin Fc region 6 including a hinge region, and full-length human endostatin domain 4.

The term "angiogenesis inhibitor," as used herein, refers to any polypeptide chain that reduces or inhibits the formation of new blood vessels in a mammal. With regard to cancer therapy, the angiogenesis inhibitor reduces or inhibits the formation of new blood vessels in or on a tumor, preferably in or on a solid tumor. It is contemplated that useful angiogenesis inhibitors may be identified using a variety of assays well known and used in the art. Such assays include, for example, the bovine capillary endothelial cell proliferation assay, the chick chorioallantoic membrane (CAM) assay or the mouse corneal assay. However, the CAM assay is preferred (see, for example, O'Reilly *et al.* (1994) Cell 79: 315-328 and O'Reilly *et al.* (1997) Cell 88: 277-285, the disclosures of which are incorporated herein by reference). Briefly,

embryos with intact yolks are removed from fertilized three day old white eggs and placed in a petri dish. After incubation at 37°C, 3% CO_2 for three days, a methylcellulose disk containing the putative angiogenesis inhibitor is applied to the chorioallantoic membrane of an individual embryo. After incubation for about 48 hours, the chorioallantoic membranes were observed under a microscope for evidence of zones of inhibition.

Preferred angiogenesis inhibitors useful in the practice of the invention include, for example, angiostatin (O'Reilly *et al.* (1994) Cell *79*: 315-328, and U.S. Patent Nos. 5,733,876; 5,837,682; and 5,885,795), and endostatin (O'Reilly *et al.* (1997) Cell *88*: 277-285 and U.S. Patent No. 5,854,205). As stated previously, angiostatin and endostatin are specific inhibitors of endothelial cell proliferation and are capable of inhibiting tumor growth by blocking angiogenesis, the formation of new blood vessels that nourish tumors.

Angiostatin has been identified as a proteolytic fragment of plasminogen (O'Reilly *et al.* (1994) Cell *79*: 315-328, and U.S. Patent Nos. 5,733,876; 5,837,682; and 5,885,795, the disclosure of which is incorporated herein by reference). Specifically, angiostatin is a 38 kDa internal fragment of plasminogen containing at least three of the Kringle regions of plasminogen. Endostatin has been identified as a proteolytic fragment of collagen XVIII (O'Reilly et al. (1997) Cell 88: 277-285, the disclosure of which is incorporated herein by reference). Specifically, endostatin is a 20 kDa C-terminal fragment of collagen XVIII. The terms "angiostatin" and "endostatin," as used herein, refer not only to the full length proteins, but also to variants and bioactive fragments thereof, as well as to bioactive fragments of plasminogen and collagen XVIII, respectively. The term bioactive fragment, with respect to angiostatin refers to any protein fragment of plasminogen or angiostatin that has at least 30%, more preferably at least 70%, and most preferably at least 90% of the activity of full-length angiostatin as determined by the CAM assay. The term bioactive fragment, with respect to endostatin refers to any protein fragment of collagen XVIII or endostatin that has at least 30%, more preferably at least 70% and most preferably at least 90% of the activity of full length endostatin as determined by the CAM assay.

The term variants includes specifies and allelic variants, as well as other naturally occurring or non-naturally occurring variants, for example, generated by conventional genetic engineering protocols, that are at least 70% similar or 60% identical, more preferably at least

75% similar or 65% identical, and most preferably 80% similar or 70% identical to either the naturally-occurring sequences of endostatin or angiostatin disclosed herein.

To determine whether a candidate polypeptide has the requisite percentage similarity or identity to a reference polypeptide, the candidate amino acid sequence and the reference amino acid sequence are first aligned using the dynamic programming algorithm described in Smith and Waterman (1981), J. Mol. Biol. 147:195-197, in combination with the BLOSUM62 substitution matrix described in Figure 2 of Henikoff and Henikoff (1992), "Amino acid substitution matrices from protein blocks", Proc. Natl. Acad Sci. USA 89:10915-10919. For the present invention, an appropriate value for the gap insertion penalty is -12, and an appropriate value for the gap extension penalty is -4. Computer programs performing alignments using the algorithm of Smith-Waterman and the BLOSUM62 matrix, such as the GCG program suite (Oxford Molecular Group, Oxford, England), are commercially available and widely used by those skilled in the art.

Once the alignment between the candidate and reference sequence is made, a percent similarity score may be calculated. The individual amino acids of each sequence are compared sequentially according to their similarity to each other. If the value in the BLOSUM62 matrix corresponding to the two aligned amino acids is zero or a negative number, the pair-wise similarity score is zero; otherwise the pair-wise similarity score is 1.0. The raw similarity score is the sum of the pair-wise similarity scores of the aligned amino acids. The raw score then is normalized by dividing it by the number of amino acids in the smaller of the candidate or reference sequences. The normalized raw score is the percent similarity. Alternatively, to calculate a percent identity, the aligned amino acids of each sequence again are compared sequentially. If the amino acids are non-identical, the pair-wise identity score is zero; otherwise the pair-wise identity score is 1.0. The raw identity score is the sum of the identical aligned amino acids. The raw score is then normalized by dividing it by the number of amino acids in the smaller of the candidate or reference sequences. The normalized raw score is the percent identity. Insertions and deletions are ignored for the purposes of calculating percent similarity and identity. Accordingly, gap penalties are not used in this calculation, although they are used in the initial alignment.

The target proteins disclosed herein are expressed as fusion proteins with an Fc region of an immunoglobulin. As is known, each immunoglobulin heavy chain constant region is comprised of four or five domains. The domains are named sequentially as follows: CH_1-hinge-CH_2-CH_3(-CH_4). The DNA sequences of the heavy chain domains have cross-homology among the immunoglobulin classes, e.g., the CH_2 domain of IgG is homologous to the CH_2 domain of IgA and IgD, and to the CH_3 domain of IgM and IgE.

As used herein, the term, "immunoglobulin Fc region" is understood to mean the carboxyl-terminal portion of an immunoglobulin chain constant region, preferably an immunoglobulin heavy chain constant region, or a portion thereof. For example, an immunoglobulin Fc region may comprise 1) a CH_1 domain, a CH_2 domain, and a CH_3 domain, 2) a CH_1 domain and a CH_2 domain, 3) a CH_1 domain and a CH_3 domain, 4) a CH_2 domain and a CH_3 domain, or 5) a combination of two or more domains and an immunoglobulin hinge region. In a preferred embodiment the Fc region used in the DNA construct includes at least an immunoglobulin hinge region a CH_2 domain and a CH_3 domain and preferably lacks at least the CH_1 domain.

The currently preferred class of immunoglobulin from which the heavy chain constant region is derived is IgG (Igγ) (γ subclasses 1, 2, 3, or 4). Other classes of immunoglobulin, IgA (Igα), IgD (Igδ), IgE (Igε) and IgM (Igμ), may be used. The choice of appropriate immunoglobulin heavy chain constant regions is discussed in detail in U.S. Patent Nos. 5,541,087, and 5,726,044. The choice of particular immunoglobulin heavy chain constant region sequences from certain immunoglobulin classes and subclasses to achieve a particular result is considered to be within the level of skill in the art. The portion of the DNA construct encoding the immunoglobulin Fc region preferably comprises at least a portion of a hinge domain, and preferably at least a portion of a CH_3 domain of Fcγ or the homologous domains in any of IgA, IgD, IgE, or IgM.

Depending on the application, constant region genes from species other than human e.g., mouse or rat may be used. The Fc region used as a fusion partner in the immunofusin DNA construct generally may be from any mammalian species. Where it is undesirable to elicit an immune response in the host cell or animal against the Fc region, the Fc region may be derived from the same species as the host cell or animal. For example, human Fc can be used when the

host animal or cell is human; likewise, murine Fc can be used where the host animal or cell will be a mouse. Further, substitution or deletion of constructs of these constant regions, in which one or more amino acid residues of the constant region domains are substituted or deleted also would be useful. One example would be to introduce amino acid substitutions in the upper CH_2 region to create a Fc variant with reduced affinity for Fc receptors (Cole et al. (1997) J. Immunol. 159:3613). One of ordinary skill in the art can prepare such constructs using well known molecular biology techniques.

The use of human Fcγ1 as the Fc region sequence has several advantages. For example, if the angiogenesis inhibitor Fc fusion protein is to be used as a biopharmaceutical, the Fcγ1 domain may confer the effector function activities to the fusion protein. The effector function activities include the biological activities such as complement fixation, antibody-directed cellular cytotoxicity, placental transfer, and increased serum half-life. The Fc domain also provides for detection by anti-Fc ELISA and purification through binding to *Staphylococcus aureus* protein A ("Protein A"). In certain applications, however, it may be desirable to delete specific effector functions from the Fc region, such as Fc receptor binding or complement fixation.

In the case of angiogenesis inhibitor immunofusins, one function of the immunoglobulin Fc fusion partner is to facilitate proper folding of the angiogenesis inhibitor protein to yield active angiogenesis inhibitor protein and to impact solubility to the active moieties, at least in the extracellular medium. Since the Fc fusion partner is hydrophilic, the angiogenesis inhibitor immunofusin readily is soluble unlike, for example, the recombinant endostatin produced in *E. coli* (O'Reilly (1997) Cell 88:277.) In all of the Examples disclosed herein, high levels of production of the immunofusins were obtained. The angiogenesis inhibitor immunofusins were secreted into media at concentrations typically of about 30 to 100 μg/ml, and could be purified readily to homogeneity by Protein A chromatography. In addition, the angiogenesis inhibitor immunofusins could be cleaved and further purified using conventional purification protocols using, for example, by heparin sepharose, lysine sepharose or affinity purification.

In addition to the high levels of expression, fusion proteins of the invention also exhibit longer serum half-lives, presumably due to their larger molecular sizes. For example, human Fc-human angiostatin has a serum half-life of 33 hours in mouse, as compared to 4-6 hours for human angiostatin (O'Reilly et al. (1996) Nature Medicine 2:689). It is believe that angiostatin

with a molecular weight of 40 kD, and endostatin with a molecular weight of 20 kD, are small enough to be cleared efficiently by renal filtration. In contrast, the dimeric forms of Fc-angiostatin and dimeric Fc-endostatin are 145 kD and 100 kD, respectively, because there are two immunoglobulin Fc regions attached to either two angiostatin molecules or two endostatin molecules. Such a bivalent structure may exhibit a higher binding affinity to the angiostatin or endostatin receptor. If the angiogenesis inhibiting activity is receptor-mediated, the Fc fusion proteins are potentially more effective to suppress tumors than monovalent angiostatin or monovalent endostatin by themselves. Furthermore, if angiostatin and/or endostatin belong to a class of dimeric protein ligands, the physical constraint imposed by the Fc on angiostatin or endostatin would make the dimerization an intramolecular process, thus shifting the equilibrium in favor of the dimer and enhancing its binding to the receptor. Cysteine residues can also be introduced by standard recombinant DNA technology to the monomer at appropriate places to stabilize the dimer through covalent disulfide bond formation.

As used herein, the term "multivalent" refers to a recombinant molecule that incorporates two or more biologically active segments. The protein fragments forming the multivalent molecule may be linked through a polypeptide peptide linker which attaches the constituent parts without causing a frame shift and permits each to function independently.

As used herein, the term "bivalent" refers to a multivalent recombinant molecule having two target proteins in a fusion construct of the invention, e.g., an Fc-X molecule, where X independently is selected from angiostatin, endostatin, or a variant thereof. Since there are two X moieties fused to an immunoglobulin Fc region (which typically itself is a dimer of the heavy chain fragments including at least a portion of the hinge region and CH_3 domain, and optionally the CH_2 domain), the molecule is bivalent (see, e.g., Figure 1A). If the fusion construct of the invention has the form Fc-X-X, the resulting Fc dimer molecule is tetravalent. The two proteins forming the Fc-X-X molecule may be linked through a peptide linker. A bivalent molecule can increase the apparent binding affinity between the molecule and its receptor. For instance, if one endostatin moiety of an Fc-endostatin can bind to a receptor on a cell with a certain affinity, the second endostatin moiety of the same Fc-endostatin may bind to a second receptor on the same cell with a much higher avidity (apparent affinity). This is because of the physical proximity of the second endostatin moiety to the receptor after the first endostatin moiety is already bound. In the case of an antibody binding to an antigen, the apparent affinity is increased by at least 10^4.

As used herein, the terms "multimer" and "multimeric" refers to the stable association of two or more polypeptide chains either covalently, for example, by means of covalent interaction, for example, by a disulfide bond or non-covalently, for example, by hydrophobic interaction. The term multimer is intended to encompass both homomultimers, wherein the polypeptides are the same, as well as heteromultimers, wherein the polypeptides are different.

As used herein, the term "dimeric" refers to a specific multimeric molecule where two protein polypeptide chains are stably associated through covalent or non-covalent interactions. It should be understood that the immunoglobulin Fc region Fc fragment itself typically is a dimer of the heavy chain fragments including at least a portion of the hinge region and CH_3 domain, and optionally the CH_2 domain. Many protein ligands are known to bind to their receptors as a dimer. If a protein ligand X dimerizes naturally, the X moiety in an Fc-X molecule will dimerize to a much greater extent, since the dimerization process is concentration dependent. The physical proximity of the two X moieties connected by associated immunoglobulin Fc region would make the dimerization an intramolecular process, greatly shifting the equilibrium in favor of the dimer and enhancing its binding to the receptor.

It is understood that the present invention exploits conventional recombinant DNA methodologies for generating the Fc fusion proteins useful in the practice of the invention. The Fc fusion constructs preferably are generated at the DNA level, and the resulting DNAs integrated into expression vectors, and expressed to produce the immunofusins. As used herein, the term "vector" is understood to mean any nucleic acid comprising a nucleotide sequence competent to be incorporated into a host cell and to be recombined with and integrated into the host cell genome, or to replicate autonomously as an episome. Such vectors include linear nucleic acids, plasmids, phagemids, cosmids, RNA vectors, viral vectors and the like. Non-limiting examples of a viral vector include a retrovirus, an adenovirus and an adeno-associated virus. As used herein, the term "gene expression" or "expression" of a target protein, is understood to mean the transcription of a DNA sequence, translation of the mRNA transcript, and secretion of an Fc fusion protein product.

A useful expression vector is pdCs (Lo et al. (1988) Protein Engineering 11:495, the disclosure of which is incorporated herein by reference) in which the transcription of the Fc-X gene utilizes the enhancer/promoter of the human cytomegalovirus and the SV40

polyadenylation signal. The enhancer and promoter sequence of the human cytomegalovirus used was derived from nucleotides -601 to +7 of the sequence provided in Boshart et al., 1985, Cell 41:521, the disclosure of which is incorporated herein by reference. The vector also contains the mutant dihydrofolate reductase gene as a selection marker (Simonsen and Levinson (1983) Proc. Nat. Acad. Sci. USA 80:2495, the disclosure of which is incorporated herein by reference).

An appropriate host cell can be transformed or transfected with the DNA sequence of the invention, and utilized for the expression and secretion of a target protein. Currently preferred host cells for use in the invention include immortal hybridoma cells, NS/O myeloma cells, 293 cells, Chinese hamster ovary cells, Hela cells, and COS cells.

The fusion proteins of the invention preferably are generated by conventional recombinant DNA methodologies. The fusion proteins preferably are produced by expression in a host cell of a DNA molecule encoding a signal sequence, an immunoglobulin Fc region and a target protein (also referred to herein as an angiogenesis inhibitor). Preferred constructs may encode in a 5' to 3' direction, the signal sequence, the immunoglobulin Fc region and the target protein. Alternatively, the constructs may encode in a 5' to 3' direction, the signal sequence, the target protein and the immunoglobulin Fc region.

As used herein, the term "signal sequence" is understood to mean a peptide segment which directs the secretion of the angiogenesis inhibitor immunofusin protein and is thereafter cleaved following translation in the host cell. The signal sequence of the invention is a polynucleotide, which encodes an amino acid sequence that initiates transport of a protein across the membrane of the endoplasmic reticulum. Signal sequences which will be useful in the invention include antibody light chain signal sequences, e.g., antibody 14.18 (Gillies et. al., 1989, Jour. of Immunol. Meth., 125:191-202), antibody heavy chain signal sequences, e.g., the MOPC141 antibody heavy chain signal sequence (Sakano et al., 1980, Nature 286:5774), and any other signal sequences which are known in the art (see for example, Watson, 1984, Nucleic Acids Research 12:5145). Each of these references is incorporated herein by reference.

Signal sequences have been well characterized in the art and are known typically to contain 16 to 30 amino acid residues, and may contain greater or fewer amino acid residues. A typical

signal peptide consists of three regions: a basic N-terminal region, a central hydrophobic region, and a more polar C-terminal region. The central hydrophobic region contains 4 to 12 hydrophobic residues that anchor the signal peptide across the membrane lipid bilayer during transport of the nascent polypeptide. Following initiation, the signal peptide is usually cleaved within the lumen of the endoplasmic reticulum by cellular enzymes known as signal peptidases. Potential cleavage sites of the signal peptide generally follow the "(-3, -1) rule." Thus a typical signal peptide has small, neutral amino acid residues in positions -1 and -3 and lacks proline residues in this region. The signal peptidase will cleave such a signal peptide between the -1 and +1 amino acids. Thus, the portion of the DNA encoding the signal sequence may be cleaved from the amino-terminus of the immunofusin protein during secretion. This results in the secretion of a immunofusin protein consisting of the Fc region and the target protein. A detailed discussion of signal peptide sequences is provided by von Heijne (1986) Nucleic Acids Res., 14:4683 the disclosure of which is incorporated herein by reference.

As would be apparent to one of skill in the art, the suitability of a particular signal sequence for use in the invention may require some routine experimentation. Such experimentation will include determining the ability of the signal sequence to direct the secretion of an immunofusin and also a determination of the optimal configuration, genomic or cDNA, of the sequence to be used in order to achieve efficient secretion of immunofusins. Additionally, one skilled in the art is capable of creating a synthetic signal peptide following the rules presented by von Heijne, referenced above, and testing for the efficacy of such a synthetic signal sequence by routine experimentation. A signal sequence may also be referred to as a "signal peptide," "leader sequence," or "leader peptide."

The fusion of the signal sequence and the immunoglobulin Fc region is sometimes referred to herein as secretion cassette. An exemplary secretion cassette useful in the practice of the invention is a polynucleotide encoding, in a 5' to 3' direction, a signal sequence of an immunoglobulin light chain gene and an Fcγ1 region of the human immunoglobulin γ1 gene. The Fcγ1 region of the immunoglobulin Fcγ1 gene preferably includes at least a portion of the hinge domain and at least a portion of the CH_3 domain, or alternatively at least portions of the hinge domain, CH_2 domain and CH_3 domain. The DNA encoding the secretion cassette can be in its genomic configuration or its cDNA configuration.

In another embodiment, the DNA sequence encodes a proteolytic cleavage site interposed between the secretion cassette and the angiogenesis inhibitor protein. A cleavage site provides for the proteolytic cleavage of the encoded fusion protein thus separating the Fc domain from the angiogenesis inhibitor protein. As used herein, "proteolytic cleavage site" is understood to mean amino acid sequences which are preferentially cleaved by a proteolytic enzyme or other proteolytic cleavage agents. Useful proteolytic cleavage sites include amino acids sequences which are recognized by proteolytic enzymes such as trypsin, plasmin or enterokinase K. Many cleavage site/cleavage agent pairs are known. See, for example, U.S. Patent No. 5,726,044, the disclosure of which is incorporated herein by reference. Where the target protein sequence is a precursor molecule to angiostatin, endostatin, or an active variant thereof, the desired protein product may be produced by cleavage with the endogenous proteolytic enzyme, such as elastin or plasmin or urokinase.

The present invention also encompasses fusion proteins containing different combinations of recombinant angiostatin and endostatin, or fragments thereof, which can be made in large quantities. Despite the demonstrated efficacy in suppressing tumor growth, the mechanism of how angiostatin and endostatin block angiogenesis is not completely known. Angiostatin has several Kringle structures and endostatin has different structural motifs, each of which may be solely responsible for or assist in binding of the proteins to endothelial cells and exerting an anti-angiogenic effect. Accordingly, this invention includes target proteins which are bioactive fragments of angiostatin, such as Kringle 1, Kringle 2, Kringle 3, and combinations thereof, and endostatin which exhibit physiologically similar behavior to naturally occurring full-length angiostatin and endostatin.

Another embodiment of the present invention provides for bifunctional hybrid constructs of angiogenesis inhibitors. As used herein, a bifunctional hybrid molecule or construct means a protein produced by combining two protein subunits, where the two subunits can be derived from different proteins. Each protein subunit has its own independent function so that in the hybrid molecule, the functions of the two subunits may be additive or synergistic. Such functional hybrid proteins would allow the synergistic effect of angiostatin and endostatin to be explored in animal models. A preferred bifunctional hybrid may comprise at least two different angiogenesis inhibitors linked in tandem, either directly or by means of a polypeptide linker. For example, in a preferred embodiment, the target sequence encodes at least a portion of angiostatin linked in

frame with at least a portion of endostatin and both the angiostatin and endostatin domains exhibit anti angiogenesis activity or angiogenesis inhibition. The two units may be linked by a polypeptide linker.

As used herein the term "polypeptide linker is understood to mean an peptide sequence that can link two proteins together or a protein and an Fc region. The polypeptide linker preferably comprises a plurality of amino acids such as glycine and/or serine. Preferably, the polypeptide linker comprises a series of glycine and serine peptides about 10-15 residues in length. See, for example, U.S. Patent No. 5,258,698, the disclosure of which is incorporated herein by reference. It is contemplated however, that the optimal linker length and amino acid composition may be determined by routine experimentation.

It is found that when different parts of the angiostatin are expressed as Fc fusion molecules, high levels of expression are obtained, presumably because the Fc portion acts as a carrier, helping the polypeptide at the C-terminus to fold correctly. In addition, the Fc region can be glycosylated and highly charged at physiological pH, thus the Fc region can help to solubilize hydrophobic proteins.

The present invention also provides methods for the production of angiostatin and endostatin of non-human species as Fc fusion proteins. Non-human angiogenesis inhibitor fusion proteins are useful for preclinical studies of angiogenesis inhibitors because efficacy and toxicity studies of a protein drug must be performed in animal model systems before testing in humans. A human protein may not work in a mouse model because the protein may elicit an immune response, and/or exhibit different pharmacokinentics skewing the test results. Therefore, the equivalent mouse protein is the best surrogate for the human protein for testing in a mouse model.

The standard Lewis lung carcinoma model in mice (O'Reilly et al. (1997) Cell 88:277) was used to compare soluble huFc-huAngiostatin, huFc-huEndostatin, muFc-muAngiostatin, muFc-muEndostatin with the insoluble proteins produced in an *E. coli* expression system. The soluble Fc fusion proteins were more efficacious in suppressing tumor growth in the Lewis lung model than the corresponding proteins produced in E. coli. Furthermore, laboratory mice are inbred and their tumors are induced and not spontaneous. Therefore, efficacy in a mouse model

may not correlate to probable efficacy against human tumors. Preclinical studies in dogs will provide more precise information about the efficacy of these angiogenesis inhibitors on spontaneous tumors because there are numerous naturally occurring, spontaneous canine tumors. The methods of producing murine (mu) Fc-mu angiostatin, muFc-mu endostatin, and canine (ca) Fc-ca angiostatin, caFc-ca endostatin of the present invention will facilitate preclinical studies of angiogenesis inhibitors in both murine and canine systems.

The present invention provides methods of treating a condition mediated by angiogenesis by administering the DNA, RNA or proteins of the invention. Conditions mediated by angiogenesis include, for example: solid tumors; blood born tumors, tumor metastasis, benign tumors including hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyrogenic granulomas; rheumatoid arthritis; psoriasis; ocular angiogenic diseases (diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma) retrolental fibroplasia, rubeosis, Osler-Webber Syndrome; myocardial angiogenesis; plaque neovascularization; telangiectasia; hemophiliac joints' angiofibroma; and wound granulation; and excessive or abnormal stimulation of endothelial cells, intestinal adhesions, artherosclerosis, sclerodermal and hypertrophic scars, i.e., keloids.

The DNA constructs disclosed herein can be useful in gene therapy procedures in which the endostatin or angiostatin gene is delivered into a cell by one of various means e.g., native DNA associated with a promoter or DNA within a viral vector. Once inside a cell, the angiostatin and/or endostatin gene or gene fragment is expressed and the protein is produced *in vivo* to carry out its normal biological function. The DNA construct of the present invention results in high levels of expression of the fusion protein. The fusion proteins of the present invention may also be useful in treating conditions mediated by angiogenesis and may have greater clinical efficacy than native angiogenesis inhibitors and other recombinant angiogenesis inhibitors because the angiogenesis inhibitor immunofusins of the present invention have a longer serum half-life than the other recombinant angiogenesis inhibitors or native angiogenesis inhibitors alone. The bivalent and dimeric forms of the present invention should have higher binding affinity due to the bivalent and dimeric structure. The bifunctional hybrid molecules of the present invention may have a higher clinical efficacy due to possible synergistic effects of two different angiogenesis inhibitors connected by the fused Fc region or a flexible polypeptide linker.

The compositions of the present invention may be provided to an animal by any suitable means, directly (e.g., locally, as by injection, implantation or topical administration to a tissue locus) or systemically (e.g., parenterally or orally). Where the composition is to be provided parenterally, such as by intravenous, subcutaneous, ophthalmic, intraperitoneal, intramuscular, buccal, rectal, vaginal, intraorbital, intracerebral, intracranial, intraspinal, intraventricular, intrathecal, intracisternal, intracapsular, intranasal or by aerosol administration, the composition preferably comprises part of an aqueous or physiologically compatible fluid suspension or solution. Thus, the carrier or vehicle is physiologically acceptable so that in addition to delivery of the desired composition to the patient, it does not otherwise adversely affect the patient's electrolyte and/or volume balance. The fluid medium for the agent thus can comprise normal physiologic saline (e.g., 9.85% aqueous NaCl, 0.15 M, pH 7-7.4).

Preferred dosages of the immunofusins per administration are within the range of 50 ng/m^2 to 1 g/m^2, more preferably 5 μg/m^2 to 200 mg/m^2, and most preferably 0.1 mg/m^2 to 50 mg/m^2. Preferred dosages of nucleic acids encoding the immunofusins per administration are within the range of 1 μg/m^2 to 100 mg/m^2, more preferably 20 μg/m^2 to 10 mg/m^2, and most preferably 400 μg/m^2 to 4 mg/m^2. It is contemplated, however, that the optimal modes of administration, and dosages may be determined by routine experimentation well within the level of skill in the art.

The invention is illustrated further by the following non-limiting examples.

EXAMPLES

Example 1. Expression of huFc-huEndostatin

Human endostatin was expressed as a human Fc-human endostatin (huFc-huEndo) fusion protein according to the teachings of Lo et al. (1998) Protein Engineering 11:495. Fc refers to the Fc fragment of the human immunoglobulin gamma (DNA sequence set forth in SEQ ID NO:1; amino acid sequence set forth in SEQ ID NO:2). (Polymerase chain reactions PCR) was used to adapt the endostatin cDNA (SEQ ID NO:3; whose amino acid sequence is disclosed in SEQ ID NO:4), for expression in an Fc-Endo fusion protein. The forward primer was either 5'-CC CCG GGT AAA CAC AGC CAC CGC GAC TTC C (SEQ ID NO:5; encoded amino acids disclosed in SEQ ID NO:6) or 5'-C AAG CTT CAC AGC CAC CGC GAC TTC C (SEQ ID NO:7; encoded amino acids disclosed in SEQ ID NO:8), where the XmaI site or the HindIII site

was followed by sequence encoding the N-terminus of endostatin. The primer with the XmaI site adapted the endostatin cDNA for ligation to the XmaI site at the end of the CH_3 domain of the IgGFc region. The primer with the HindIII site adapted the endostatin cDNA for ligation to the HindIII site of the pdCs-Fc(D_4K) vector, which contains the enterokinase recognition site Asp_4-Lys (LaVallie et al. (1993) J. Biol. Chem. 268:23311-23317) at the junction of the fusion protein. The reverse primer was 5'-C CTC GAG CTA CTT GGA GGC AGT CAT G (SEQ ID NO:9), which was designed to put a translation STOP codon (anticodon, CTA) immediately after the C-terminus of endostatin, and this was followed by an XhoI site. The PCR products were cloned and sequenced, and the XmaI-XhoI fragment was ligated to the resulting XmaI and XhoI digested pdCs-Fc vector. Similarly, the HindIII-XhoI fragment encoding endostatin was ligated into appropriately digested pdCs-huFc(D_4K) vector. Stable clones expressing Fc-endo or Fc(D_4K)-endostatin were obtained by electroporation of NS/0 cells followed by selection in growth medium containing 100 nM methotrexate. Protein expression level was assayed by anti-human Fc ELISA (Example 3) and confirmed by SDS-PAGE, which showed a protein product of ~52 kD. The best producing clones were subcloned by limiting dilutions.

Example 2. Cell culture and transfection

For transient transfection, the plasmid was introduced into human kidney 293 cells by co-precipitation of plasmid DNA with calcium phosphate (Sambrook et al. (1989) Molecular Cloning - A Laboratory Manual, Cold Spring Harbor, NY) or by lipofection using LipofectAMINE Plus (Life Technologies, Gaithersburg, MD) according to supplier's protocol.

In order to obtain stably transfected clones, plasmid DNA was introduced into the mouse myeloma NS/0 cells by electroporation. NS/0 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. About 5 x 10^6 cells were washed once with PBS and resuspended in 0.5 ml PBS. Ten μg of linearized plasmid DNA then was incubated with the cells in a Gene Pulser Cuvette (0.4 cm electrode gap, BioRad, Hercules, CA) on ice for 10 min. Electroporation was performed using a Gene Pulser (BioRad, Hercules, CA) with settings at 0.25 V and 500 μF. Cells were allowed to recover for 10 min. on ice, after which they were resuspended in growth medium and then plated onto two 96 well plates. Stably transfected clones were selected by growth in the presence of 100 nM methotrexate (MTX), which was introduced two days post-transfection. The cells were fed every 3 days for three more times, and

MTX-resistant clones appeared in 2 to 3 weeks. Supernatants from clones were assayed by anti-Fc ELISA to identify high producers. High producing clones were isolated and propagated in growth medium containing 100 nM MTX.

Example 3. ELISA Procedures

Three different ELISAs were used to determine the concentrations of protein products in the supernatants of MTX-resistant clones and other test samples. The anti-human Fc (huFc) ELISA was used to measure the amount of human Fc-containing proteins. The anti-murine Fc (muFc) and anti-canine Fc (caFc) antibodies were used in ELISAs to measure the amount of murine Fc- and canineFc-containing proteins, respectively. The procedure for the anti-huFc ELISA is described in detail herein below.

A. Coating plates

ELISA plates were coated with AffiniPure Goat anti-Human IgG (H+L) (Jackson ImmunoResearch Laboratories, West Grove, PA) at 5 μg/ml in PBS, and 100 μl/well in 96-well plates (Nunc-Immuno plate MaxiSorp™, Nalge Nunc International, Rochester, NY). Coated plates were covered and incubated at 4°C overnight. Plates then were washed 4 times with 0.05% Tween 20 in PBS, and blocked with 1% BSA/1% Goat Serum in PBS, 200 μl/well. After incubation with the blocking buffer at 37°C for 2 hours, the plates were washed 4 times with 0.05% Tween in PBS and tapped dry on paper towels.

B. Incubation with test samples and secondary antibody

Test samples were diluted to the proper concentrations in a sample buffer, containing 1% BSA/1% Goat Serum/0.05% Tween in PBS. A standard curve was prepared with a chimeric antibody (with a human Fc), the concentration of which was known. To prepare a standard curve, serial dilutions were made in the sample buffer to give a standard curve ranging from 125 ng/ml to 3.9 ng/ml. The diluted samples and standards were added to the plate, 100 μl/well and the plate was then incubated at 37°C for 2 hr. After incubation, the plate was washed 8 times with 0.05% Tween in PBS. To each well was then added 100 μl of secondary antibody, the horse radish peroxidase (HRP)-conjugated anti-human IgG (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA), diluted about 1:120,000 in sample buffer. The exact dilution of the secondary antibody had to be determined for each lot of the HRP-conjugated Anti-

Human IgG. After incubation at 37°C for 2 hr, the plate was washed 8 times with 0.05% Tween in PBS.

C. Development

A substrate solution was prepared by dissolving 30 mg (1 tablet) of o-phenylenediamine dihydrochloride (OPD) into 15 ml of 0.025 M citric acid/0.05 M Na_2HPO_4 buffer, pH 5, containing 0.03% of freshly added H_2O_2. The substrate solution was added to the plate at 100 µl/well. The color was allowed to develop for 30 min. at room temperature in the dark. The developing time can be subject to change, depending on lot to lot variability of the coated plates, the secondary antibody, etc. The reaction was stopped by adding 4N H_2SO_4, 100 µl/well. The plate was read by a plate reader, which was set at both 490 and 650 nm, and programmed to subtract the background OD at 650 nm from the OD at 490 nm.

The procedure for the anti-muFc ELISA was similar, except that ELISA plate was coated with AffiniPure Goat anti-murine IgG (H+L) (Jackson ImmunoResearch, West Grove, PA) at 5 µg/ml in PBS, and 100 µl/well; and the secondary antibody was horse radish peroxidase-conjugated goat anti-muIgG, Fcγ (Jackson ImmunoResearch West Grove, PA), used at 1 in 5000 dilution. Similarly, for the anti-caFc ELISA, the ELISA plate was coated with AffiniPure Rabbit anti-dog IgG, Fc Fragment specific (Jackson ImmunoResearch, West Grove, PA) at 5 µg/ml in PBS, and 100 µl/well; and the secondary antibody was horse radish peroxidase-conjugated AffiniPure rabbit anti-dog IgG, Fc fragment specific (Jackson ImmunoResearch, West Grove, PA), used at 1 in 5000 dilution.

Example 4. Expression of huFc-huAngiostatin

Human angiostatin (DNA sequence set forth in SEQ ID NO:10; amino acid sequence set forth in SEQ ID NO:11) was expressed as a human Fc-human angiostatin (huFc-huAngio) fusion protein essentially as described in Example 1. PCR was used to adapt the angiostatin cDNA (SEQ ID NO:3), for expression in the pdCs-huFc or pdCs-huFc(D_4K) vectors. The respective forward primers were 5'-CC CCG GG T AAG AAA GTG TAT CTC TCA GAG (SEQ ID NO 12; encoded amino acids disclosed in SEQ ID NO:13), and 5'- C CCC AAG CTT AAA GTG TAT CTC TCA GAG (SEQ ID NO:14; encoded amino acids disclosed in SEQ ID NO:15), where the XmaI site or the HindIII site was followed by sequence encoding the N-terminus of

angiostatin. The reverse primer was 5'-CCC CTC GAG CTA CGC TTC TGT TCC TGA GCA (SEQ ID NO:16), which was designed to put a translation STOP codon (anticodon, CTA) immediately after the C-terminus of angiostatin, and this was followed by an XhoI site. The PCR products were cloned and sequenced, and the resulting XmaI-XhoI fragment and the HindIII-XhoI fragment encoding angiostatin were ligated to the pdCs-huFc and the pdCs-huFc(D_4K) vectors, respectively. Stable NS/0 clones expressing huFc-huAngio and huFc(D_4K)-huAngio were selected and assayed as described in Examples 2 and 3.

Example 5. Expression of muFc-mu-Endostatin

Murine endostatin (DNA sequence set forth in SEQ ID NO:17; amino acid sequence set forth in SEQ ID NO:18) and murine Fc (DNA sequence set forth in SEQ ID NO:19; encoded amino acids set forth in SEQ ID NO:20) were expressed as a murine Fc-murine endostatin (muFc-muEndo) fusion protein essentially as described in Example 1. PCR was used to adapt the endostatin cDNA (SEQ ID NO:4), for expression in the pdCs-muFc(D_4K) vector. The forward primer was 5'-C CCC AAG CTT CAT ACT CAT CAG GAC TTT C (SEQ ID NO:21; encoded amino acids disclosed in SEQ ID NO:22), where the HindIII site was followed by sequence encoding the N-terminus of endostatin. The reverse primer was 5'-CCC CTC GAG CTA TTT GGA GAA AGA GGT C (SEQ ID NO:23), which was designed to put a translation STOP codon (anticodon, CTA) immediately after the C-terminus of endostatin, and this was followed by an XhoI site. The PCR product was cloned and sequenced, and the resulting HindIII-XhoI fragment encoding endostatin was ligated into the pdCs-muFc(D_4K) vector. Stable NS/0 clones expressing muFc(D_4K)-muEndo were selected and assayed (anti-muFc ELISA) as described in Examples 2 and 3.

Example 6. Expression of muFc-muAngiostatin

Murine angiostatin (DNA sequence set forth in SEQ ID NO:24; amino acid sequence set forth in SEQ ID NO:25) was expressed as a murine Fc-murine angiostatin (muFc-muAngio) fusion protein essentially as described in Example 1. PCR was used to adapt the angiostatin cDNA (SEQ ID NO:6) for expression in the pdCs-Fc(D_4K) vector. The forward primer was 5'-C CCC AAG CTT GTG TAT CTG TCA GAA TGT AAG CCC TCC TGT CTC TGA GCA (SEQ ID NO: 26; encoded amino acids disclosed in SEQ ID NO:27), where the HindIII site was

followed by sequence encoding the N-terminus of angiostatin. The reverse primer was 5'-CCC CTC GAG CTA CCC TCC TGT CTC TGA GCA (SEQ ID NO:28), which was designed to put a translation STOP codon (anticodon, CTA) immediately after the C-terminus of angiostatin, and this was followed by an XhoI site (CTCGAG). The PCR product was cloned and sequenced, and the HindIII-XhoI fragment encoding angiostatin was ligated to the pdCs-muFc(D_4K) vector. Stable NS/0 clones expressing muFc(D_4K)-muAngio were selected and assayed (anti-muFc ELISA) as described in Examples 2 and 3.

Example 7. Expression of canine Fc (caFc)

Canine peripheral blood monocytic cells (PBMCs) isolated from dog's blood were used to prepare mRNA. After synthesis of the first strand cDNA with reverse transcriptase and oligo(dT), PCR was performed to amplify the canine Fc (Kazuhiko et al., (1992) JP 1992040894-A1) using the forward primer 5'-CC TTA AGC GAA AAT GGA AGA GTT CCT CGC (SEQ ID NO:29; encoded amino acids disclosed in SEQ ID NO:30), in which an AflII site was introduced immediately upstream of the sequence encoding the hinge region of the canine Fc, and the reverse primer 5'-C CTC GAG TCA TTT ACC CGG GGA ATG GGA GAG GGA TTT CTG (SEQ ID NO:31), in which an XhoI site was introduced after the translation STOP codon (anticodon, TCA) of the canine Fc. The reverse primer also introduced a silent mutation to create a XmaI restriction site, which facilitates the construction of the pdCs-caFc(D_4K) vector through a linker-adaptor and ligation to DNA constructs encoding canine endostatin or angiostatin. Similar to the construction of pdCs-huFc, which was described in detail in Lo et al. (Lo et al., Protein Engineering (1998) 11:495), the expression vector for the pdCs-caFc was constructed as follows. The AflII-XhoI fragment encoding the canine Fc was ligated to the XbaI-AflII fragment encoding the light chain signal peptide and the XbaI-XhoI digested pdCs vector. The resulting pdCs-caFc expression vector then was used to transfect 293 cells. About 3 days post-transfection, the supernatant was purified by Protein A chromatography. Expression of dog Fc (DNA sequence set forth in SEQ ID NO:32; amino acid sequence set forth in SEQ ID NO:33) was confirmed by SDS-PAGE followed by Western blot analysis using a peroxidase-conjugated Rabbit anti-Dog IgG, Fc fragment specific (Jackson ImmunoResearch, West Grove, PA).

Example 8. Expression of caFc-caEndostatin.

The coding sequence for canine endostatin (DNA sequence set forth in SEQ ID NO:34; amino acid sequence set forth in SEQ ID NO:35) was adapted to a HindIII-XhoI fragment for expression as a Fc fusion protein, essentially as described in Example 5. At the 3' end, a STOP codon was introduced, for example, by PCR, immediately after the codon encoding the C-terminal lysine residue, and this was followed by the NotI restriction site. At the 5' end, however, there was a DraIII restriction site convenient for reconstruction. An oligonucleotide duplex consisting of a HindIII and a DraIII sticky ends was chemically synthesized and used to ligate to the DraIII-XhoI restriction fragment which encodes the rest of the canine endostatin cDNA. The duplex used is shown below:

```
HindIII
5'-AGCTT CAC ACC CAC CAG GAC TTC CAG CCG GTG CTG CAC CTG (SEQ ID NO:36)
A GTG TGG GTG GTC CTG AAG GTC GGC CAC GAC GTG-5'     (SEQ ID NO:38)
                                                       DraIII
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The first CAC in the duplex encodes the N-terminal histidine residue of the canine endostatin. The HindIII-XhoI fragment encoding the full-length canine endostatin thus could be ligated to the HindIII-XhoI digested pdCs-caFc vector (see Example 7) for expression. Stable NS/0 clones expressing caFc-caEndo were selected and assayed by anti-caFc ELISA, as described in Examples 2 and 3. The protein product was analyzed on SDS-PAGE and confirmed by Western blot analysis.

Example 9. Expression of caFc-caAngiostatin

The cDNA encoding the full length canine angiostatin (DNA sequence set forth in SEQ ID NO:39; amino acid sequence set forth in SEQ ID NO:40) was adapted for expression as a caFc fusion protein essentially as in the aforementioned examples. Briefly, at the 3' end, a STOP codon was introduced, for example, by PCR, immediately after the codon encoding the C-terminal lysine residue and this was followed by a NotI restriction site instead of an XhoI site, since there was an internal XhoI restriction site in the cDNA of the canine angiostatin. At the 5' end, a HindIII site was introduced in-frame immediately upstream of the N-terminus of angiostatin. The HindIII-NotI fragment encoding the full length canine angiostatin then was ligated to the HindIII-NotI digested pdCs-caFc vector (where the NotI site was introduced at the

XhoI site through linker ligation) for expression. Stable NS/0 clones expressing caFc-caAngio were selected and assayed by anti-caFc ELISA, as described in Examples 2 and 3. The protein product was analyzed on SDS-PAGE and confirmed by Western blot analysis.

Example 10. Expression of muFc-K1 of muAngio

Angiostatin comprises the first four of the five Kringle domains of plasminogen. To determine if any one or several Kringle domains are responsible for the observed anti-angiogenic activity of angiostatin, it is possible to produce single Kringle domains by themselves or combination thereof for testing. To demonstrate the utility of Fc as a fusion protein partner, the expression of the first Kringle domain of murine angiostatin (K1) was achieved in the following way. The first Kringle domain ends at Glu-87 of murine angiostatin (SEQ ID NO:25). There was a convenient NsiI restriction site in the cDNA at this position so that after digestion by NsiI, the four-base 3'-overhang was removed by T4 polymerase to create a blunt end. A translation STOP codon was introduced immediately downstream of the GAA encoding Glu-87 via ligation to the palindromic linker TGA CTC GAG TCA (SEQ ID NO: 41), where the STOP codon TGA was followed by an XhoI site. The HindIII-XhoI fragment encoding this truncated angiostatin, i.e., first Kringle only, then was ligated into the pdCs-muFc(D_4K) vector for expression. High levels of expression were obtained in both transient and stable expression, as analyzed by anti-muFc ELISA and SDS-PAGE.

Example 11. Expression of muFc-innerK1 of muAngio

A Kringle domain consists of multiple loops, including an outer loop and an inner loop. In the first Kringle of murine angiostatin, the inner loop is defined by Cys 55 and Cys 79, which together form a disulfide bond at the base of the loop. The Cys-67 of the inner loop forms another disulfide bond with a Cys residue of the outer loop to give the Kringle structure. To test if the inner loop has any anti-angiogenic activity, it was expressed as a muFc-inner K1 (Kringle 1) as follows. With a DNA fragment encoding the first Kringle as template, a mutagenic primer having the sequence 5'GGG CCT TGG AGC TAC ACT ACA (SEQ ID NO: 42; encoded amino acids disclosed in SEQ ID NO:43) was used to mutagenize TGC (Cys-67) to AGC (Ser), by PCR. This ensures that the Cys-67 does not form a disulfide bond when the inner loop of Kringle 1 is expressed without the outer loop. An upstream primer having the sequence

5'GCGGATCCAAGCTT AGT ACA CAT CCC AAT GAG GG (SEQ ID NO:44; encoded amino acids disclosed in SEQ ID NO:45) was used to introduce a HindIII site in frame immediately 5' to the codon for Ser-43 (AGT). A BamHI site was also introduced immediately upstream of the HindIII site. The BamHI site is useful for ligating to the BamHI site at the end of the flexible Gly-Ser linker shown in Example 12 below. Thus a HindIII-XhoI DNA fragment encoding Ser-43 through Glu-87 of murine angiostatin was ligated to the pdCs-muFc(D_4K) vector for expression. High levels of expression of muFc-innerK1 were obtained in both transient and stable expression, as analyzed by anti-muFc ELISA and SDS-PAGE.

Example 12. Expression of muFc-muEndo-GlySer linker-innerK1 of muAngio

The hybrid molecule muFc-muEndo-innerK1 comprises muFc-muEndo joined by a polypeptide linker containing glycine and serine residues, to the inner loop of the first Kringle of murine angiostatin. The DNA construct was assembled as follows.

There is a BspHI site at the 3' end of the murine endostatin cDNA. To introduce a flexible linker of glycine and serine residues at the C-terminus of murine endostatin, a 540-bp HindIII-BspHI fragment encoding endostatin was ligated to an overlapping oligonucleotide duplex formed by the oligonucleotides disclosed in SEQ ID NO:46 and SEQ ID NO:48. The amino acid linker encoded by SEQ ID NO:46 is disclosed in SEQ ID NO:47.

The HindIII-BamHI fragment encoding murine endostatin and the Gly-Ser linker was subcloned into a standard cloning vector. The BamHI site was then used to introduce the BamHI-XhoI fragment encoding the innerK1 in Example 11. The resulting HindIII-XhoI fragment encoding muEndo-GlySer linker-innerK1, was ligated to the pdCs-muFc(D_4K) vector for expression. High levels of expression of muFc-muEndo-GlySer linker-innerK1 were obtained in both transient and stable expression, as analyzed by anti-muFc ELISA and SDS-PAGE.

Example 13. Expression of muFc-muEndo-GlySer linker-K1 of muAngio

The hybrid molecule muFc-muEndo-K1 comprises muFc-muEndo joined by a polypeptide linker containing glycine and serine residues, to the first Kringle of murine angiostatin. The DNA construct was assembled as follows.

The BamHI end of the HindIII-BamHI fragment encoding the muEndo-GlySer linker (Example 12) was ligated to the HindIII-XhoI fragment encoding the Kringle 1 of murine angiostatin (Example 10) via the following adaptor:

```
BamHI
5' GA TCC TCA GGC C          (SEQ ID NO:49)
          G AGT CCG GTCGA    (SEQ ID NO:50)
                   HindIII
```

The adaptor has a HindIII' sticky end, which upon ligation, would not regenerate the HindIII site. Thus, the resulting HindIII-XhoI fragment, which encodes the muEndo-GlySer linker-Kringle 1, was ligated to the pdCs-muFc(D_4K) vector for expression. High levels of expression of muFc-muEndo-GlySer linker-K1 were obtained in both transient and stable expression, as analyzed by anti-muFc ELISA and SDS-PAGE.

Example 14 Expression of muFc-muEndo-GlySer linker-muAngio

The hybrid molecule muFc-muEndo-GlySer linker-muAngio comprises muFc-muEndo joined by a polypeptide linker containing glycine and serine residues, to murine angiostatin. The DNA construct was assembled essentially as follows. The BamHI end of the HindIII-BamHI fragment encoding the muEndo-GlySer linker (Example 12) was ligated to the HindIII-XhoI fragment encoding murine angiostatin via the adaptor described in Example 13. The resulting HindIII-XhoI fragment, which encodes the muEndo-GlySer linker-muAngio, was ligated to the pdCs-muFc(D_4K) vector for expression. High levels of expression of muFc-muEndo-GlySer linker-muAngio were obtained in both transient and stable expression, as analyzed by anti-muFc ELISA and SDS-PAGE.

Example 15. Expression of huAngio-huFc-huEndo

The hybrid molecule huAngio-huFc-huEndo comprises human angiostatin joined by a peptide bond to huFc-huEndo. The DNA construct was assembled as follows. A HindIII-XhoI fragment which encodes human angiostatin without a STOP codon was first generated by PCR, so that the codon for the last amino acid residue of angiostatin was followed immediately by CTCGAG of the XhoI site. The HindIII at the 5' end was ligated to an XbaI-AflII fragment of

the light chain signal peptide (Lo et al., Protein Engineering (1998) 11:495) via a AflII-HindIII' adaptor:

```
   AflII
5' TTA AGC GGC C               (SEQ ID NO:51)
          CG CGG GTCGA         (SEQ ID NO:52)
                  HindIII'
```

The HindIII' sticky end of the adaptor, upon ligation, would not regenerate a HindIII site. At the 3' end, the XhoI site was ligated to the AflII site of the AflII-XhoI fragment encoding the huFc-hu-Endo via the following XhoI'-AflII adaptor:

```
   XhoI'
5' TC GAC TCC GGC              (SEQ ID NO:53)
         G AGG CCG AATT        (SEQ ID NO:54)
                  AflII
```

The XhoI sticky end of the adaptor, upon ligation, would not regenerate a XhoI site. The resulting XbaI-XhoI fragment encoding the signal peptide-human angiostatin-huFc-human endostatin was cloned into the pdCs vector for expression. High levels of expression of were obtained in both transient and stable expression, as analyzed by anti-muFc ELISA and SDS-PAGE.

Example 16 Pharmacokinetics

In one set of pharmacokinetic studies, C57/BL6 mice with implanted Lewis lung tumors at 100-200 mm^3 were injected in the tail vein with 720 μg huFc-huAngio per mouse. The size of the tumors and the dosage of huFc-huAngio used in this study were chosen to simulate the actual treatment protocol described by O'Reilly (O'Reilly et al., (1996) Nature Medicine 2:689). Blood was harvested by retro-orbital bleeding at 1/2, 1, 2, 4, 8, 24, and 48 hr. post injection. The blood samples were analyzed by anti-huFc ELISA followed by Western analysis. HuFc-huAngio was found to have a circulating half-life of about 32 hr. in mouse and Western analysis showed that over 90% of the hu-Fc-huAngio remained as an intact molecule in circulation.

The pharmacokinetic studies was also repeated in Swiss mice without tumors at a dosage of 200 μg/mouse. In this case huFc-huAngio was found to have a circulating half-life of about 33 hr.

Equivalents

The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The foregoing embodiments are therefore to be considered in all respects illustrative rather than limiting on the invention described herein. Scope of the invention is thus indicated by the appended claims rather than by the foregoing description, and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced therein.

What is claimed is:

1. A DNA molecule encoding a fusion protein comprising:

(a) a signal sequence;

(b) an immunoglobulin Fc region; and

(c) a target protein sequence selected from the group consisting of angiostatin, endostatin, a plasminogen fragment having angiostatin activity, a collagen XVIII fragment having endostatin activity, and combinations thereof.

2. The DNA of claim 1 wherein said signal sequence, said immunoglobulin Fc region and said target protein sequence are encoded serially in a 5' to 3' direction.

3. The DNA of claim 1, wherein said signal sequence, said target sequence, and said immunoglobulin Fc region are encoded serially in a 5' to 3' direction.

4. The DNA of claim 1 wherein said immunoglobulin Fc region comprises an immunoglobulin hinge region.

5. The DNA of claim 1 wherein said immunoglobulin Fc region comprises an immunoglobulin hinge region and an immunoglobulin constant heavy chain domain.

6. The DNA of claim 1 wherein said immunoglobulin Fc region comprises a hinge region and an CH_3 domain.

7. The DNA of claim 1 wherein said immunoglobulin Fc region lacks at least the CH_1 domain.

8. The DNA of claim 1 wherein said immunoglobulin Fc region encodes at least a portion of immunoglobulin gamma.

9. A replicable expression vector for transfecting a mammalian cell, said vector comprising the DNA of claim 1.

10. A mammalian cell harboring the DNA of claim 1.

11. A fusion protein comprising an immunoglobulin Fc region, and a target protein selected from the group consisting of angiostatin, endostatin, a plasminogen fragment having angiostatin activity, a collagen XVIII fragment having endostatin activity, and combinations thereof.

12. The fusion protein of claim 11 wherein said plasminogen fragment has molecular weight of approximately 40 kD and comprises an amino acid sequence set forth in SEQ ID No:3.

13. The fusion protein of claim 11 wherein said target protein comprises amino acid sequence set forth in SEQ ID No:3.

14. The fusion protein of claim 11 wherein of said collagen XVIII fragment comprises the amino acid sequence set forth in SEQ ID No:1.

15. The fusion protein of claim 11 wherein said target protein comprises at least two molecules selected from the group consisting of angiostatin, endostatin, a plasminogen fragment , and a collagen XVIII fragment, wherein said two molecules are linked by a polypeptide linker.

16. The fusion protein of claim 11 wherein said target protein is linked to an N-terminal end of said immunoglobulin Fc region.

17. The fusion protein of claim 11 wherein said target protein is linked to a C-terminal end of said immunoglobulin Fc region.

18. A multimeric protein comprising at least two fusion proteins of claim 11 linked via a disulfide bond.

19. The multimeric protein of claim 18 wherein the target protein of at least one said fusion protein is angiostatin and the target protein of at least one said fusion protein is endostatin.

20. The multimeric protein of claim 18 wherein the target protein of both of said fusion proteins is angiostatin.

21. The multimeric protein of claim 18 wherein the target protein of both of said fusion proteins is endostatin.

22. The fusion protein of claim 11 further comprising a second target protein selected from the group consisting of angiostatin, endostatin, a plasminogen fragment having angiostatin activity, and a collagen XVIII fragment having endostatin activity.

23. The fusion protein of claim 22 wherein said second target protein is linked by a polypeptide linker to said first target protein.

24. The fusion protein of claim 22 wherein said first target protein is connected to an N-terminal end of said immunoglobulin Fc region and said second target protein is connected to a C-terminal end of said immunoglobulin Fc region.

25. A multimeric fusion protein comprising at least two fusion proteins of claim 11, wherein said fusion proteins are linked by a polypeptide bond.

26. A method of producing a fusion protein, the method comprising the steps of:

a) providing the mammalian cell of claim 10; and

b) culturing the mammalian cell to produce said fusion protein.

27. The method of claim 26 comprising the additional step of collecting said fusion protein.

28. The method of claim 26 comprising the additional step of cleaving said immunoglobulin Fc region from said target protein.

29. A method of treating a condition mediated by angiogenesis comprising the step of administering the DNA of claim 1 to a mammal in need of an angiogenesis inhibitor.

30. A method of treating a condition mediated by angiogenesis comprising the step of administering the vector of claim 9 to a mammal in need of an angiogenesis inhibitor.

31. A method of treating a condition alleviated by the administration of angiostatin or endostatin comprising the step of administering an effective amount of the fusion protein of claim 11 to a mammal having said condition.

SEQUENCE LISTING

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      Li, Yue
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Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val
         35                  40                  45

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            100                 105                 110

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130 135 140

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             20                  25                  30

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         35                  40                  45

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     50                  55                  60

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                 85                  90                  95
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Gly Pro Trp Cys Tyr Thr Thr Asp Pro Glu Lys Arg Tyr Asp Tyr Cys
65 70 75 80

Asp Ile Leu Glu Cys Glu Glu Glu Cys Met His Cys Ser Gly Glu Asn
85 90 95

Tyr Asp Gly Lys Ile Ser Lys Thr Met Ser Gly Leu Glu Cys Gln Ala
100 105 110

Trp Asp Ser Gln Ser Pro His Ala His Gly Tyr Ile Pro Ser Lys Phe
115 120 125

Pro Asn Lys Asn Leu Lys Lys Asn Tyr Cys Arg Asn Pro Asp Arg Glu
130 135 140

Leu Arg Pro Trp Cys Phe Thr Thr Asp Pro Asn Lys Arg Trp Glu Leu
145 150 155 160

Cys Asp Ile Pro Arg Cys Thr Thr Pro Pro Pro Ser Ser Gly Pro Thr
165 170 175

Tyr Gln Cys Leu Lys Gly Thr Gly Glu Asn Tyr Arg Gly Asn Val Ala
180 185 190

Val Thr Val Ser Gly His Thr Cys Gln His Trp Ser Ala Gln Thr Pro
195 200 205

His Thr His Asn Arg Thr Pro Glu Asn Phe Pro Cys Lys Asn Leu Asp
210 215 220

Glu Asn Tyr Cys Arg Asn Pro Asp Gly Lys Arg Ala Pro Trp Cys His
225 230 235 240

```
Thr Thr Asn Ser Gln Val Arg Trp Glu Tyr Cys Lys Ile Pro Ser Cys
                245                 250                 255

Asp Ser Ser Pro Val Ser Thr Glu Gln Leu Ala Pro Thr Ala Pro Pro
            260                 265                 270

Glu Leu Thr Pro Val Val Gln Asp Cys Tyr His Gly Asp Gly Gln Ser
        275                 280                 285

Tyr Arg Gly Thr Ser Ser Thr Thr Thr Thr Gly Lys Lys Cys Gln Ser
    290                 295                 300

Trp Ser Ser Met Thr Pro His Arg His Gln Lys Thr Pro Glu Asn Tyr
305                 310                 315                 320

Pro Asn Ala Gly Leu Thr Met Asn Tyr Cys Arg Asn Pro Asp Ala Asp
                325                 330                 335

Lys Gly Pro Trp Cys Phe Thr Thr Asp Pro Ser Val Arg Trp Glu Tyr
            340                 345                 350

Cys Asn Leu Lys Lys Cys Ser Gly Thr Glu Ala
        355                 360


<210> 12
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Forward primer
      for human Fc-Angio

<220>
<221> CDS
<222> (3)..(29)

<400> 12
cc ccg ggt aag aaa gtg tat ctc tca gag                              29
   Pro Gly Lys Lys Val Tyr Leu Ser Glu
     1               5


<210> 13
<211> 9
<212> PRT
<213> Artificial Sequence

<400> 13
Pro Gly Lys Lys Val Tyr Leu Ser Glu
  1               5


<210> 14
<211> 28
<212> DNA
<213> Artificial Sequence
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<220>
<223> Description of Artificial Sequence:Forward primer
for human Fc-Angio

<220>
<221> CDS
<222> (2)..(28)

<400> 14
c ccc aag ctt aaa gtg tat ctc tca gag 28
Pro Lys Leu Lys Val Tyr Leu Ser Glu
1 5

<210> 15
<211> 9
<212> PRT
<213> Artificial Sequence

<400> 15
Pro Lys Leu Lys Val Tyr Leu Ser Glu
1 5

<210> 16
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Reverse primer
for human Fc-Angio

<400> 16
cccctcgagc tacgcttctg ttcctgagca 30

<210> 17
<211> 552
<212> DNA
<213> Mus musculus

<220>
<221> CDS
<222> (1)..(552)
<223> endostatin

<400> 17
cat act cat cag gac ttt cag cca gtg ctc cac ctg gtg gca ctg aac 48
His Thr His Gln Asp Phe Gln Pro Val Leu His Leu Val Ala Leu Asn
1 5 10 15

acc ccc ctg tct gga ggc atg cgt ggt atc cgt gga gca gat ttc cag 96
Thr Pro Leu Ser Gly Gly Met Arg Gly Ile Arg Gly Ala Asp Phe Gln
20 25 30

tgc ttc cag caa gcc cga gcc gtg ggg ctg tcg ggc acc ttc cgg gct 144
Cys Phe Gln Gln Ala Arg Ala Val Gly Leu Ser Gly Thr Phe Arg Ala
35 40 45

```
ttc ctg tcc tct agg ctg cag gat ctc tat agc atc gtg cgc cgt gct    192
Phe Leu Ser Ser Arg Leu Gln Asp Leu Tyr Ser Ile Val Arg Arg Ala
     50                  55                  60

gac cgg ggg tct gtg ccc atc gtc aac ctg aag gac gag gtg cta tct    240
Asp Arg Gly Ser Val Pro Ile Val Asn Leu Lys Asp Glu Val Leu Ser
 65                  70                  75                  80

ccc agc tgg gac tcc ctg ttt tct ggc tcc cag ggt caa gtg caa ccc    288
Pro Ser Trp Asp Ser Leu Phe Ser Gly Ser Gln Gly Gln Val Gln Pro
                 85                  90                  95

ggg gcc cgc atc ttt tct ttt gac ggc aga gat gtc ctg aga cac cca    336
Gly Ala Arg Ile Phe Ser Phe Asp Gly Arg Asp Val Leu Arg His Pro
            100                 105                 110

gcc tgg ccg cag aag agc gta tgg cac ggc tcg gac ccc agt ggg cgg    384
Ala Trp Pro Gln Lys Ser Val Trp His Gly Ser Asp Pro Ser Gly Arg
        115                 120                 125

agg ctg atg gag agt tac tgt gag aca tgg cga act gaa act act ggg    432
Arg Leu Met Glu Ser Tyr Cys Glu Thr Trp Arg Thr Glu Thr Thr Gly
    130                 135                 140

gct aca ggt cag gcc tcc tcc ctg ctg tca ggc agg ctc ctg gaa cag    480
Ala Thr Gly Gln Ala Ser Ser Leu Leu Ser Gly Arg Leu Leu Glu Gln
145                 150                 155                 160

aaa gct gcg agc tgc cac aac agc tac atc gtc ctg tgc att gag aat    528
Lys Ala Ala Ser Cys His Asn Ser Tyr Ile Val Leu Cys Ile Glu Asn
                165                 170                 175

agc ttc atg acc tct ttc tcc aaa                                    552
Ser Phe Met Thr Ser Phe Ser Lys
            180
```

<210> 18
<211> 184
<212> PRT
<213> Mus musculus

<400> 18

```
His Thr His Gln Asp Phe Gln Pro Val Leu His Leu Val Ala Leu Asn
  1               5                  10                  15

Thr Pro Leu Ser Gly Gly Met Arg Gly Ile Arg Gly Ala Asp Phe Gln
             20                  25                  30

Cys Phe Gln Gln Ala Arg Ala Val Gly Leu Ser Gly Thr Phe Arg Ala
         35                  40                  45

Phe Leu Ser Ser Arg Leu Gln Asp Leu Tyr Ser Ile Val Arg Arg Ala
     50                  55                  60

Asp Arg Gly Ser Val Pro Il  Val Asn Leu Lys Asp Glu Val Leu Ser
 65                  70                  75                  80
```

```
Pro Ser Trp Asp Ser Leu Phe Ser Gly Ser Gln Gly Gln Val Gln Pro
                85                  90                  95

Gly Ala Arg Ile Phe Ser Phe Asp Gly Arg Asp Val Leu Arg His Pro
            100                 105                 110

Ala Trp Pro Gln Lys Ser Val Trp His Gly Ser Asp Pro Ser Gly Arg
        115                 120                 125

Arg Leu Met Glu Ser Tyr Cys Glu Thr Trp Arg Thr Glu Thr Thr Gly
    130                 135                 140

Ala Thr Gly Gln Ala Ser Ser Leu Leu Ser Gly Arg Leu Leu Glu Gln
145                 150                 155                 160

Lys Ala Ala Ser Cys His Asn Ser Tyr Ile Val Leu Cys Ile Glu Asn
                165                 170                 175

Ser Phe Met Thr Ser Phe Ser Lys
            180


<210> 19
<211> 699
<212> DNA
<213> Mus musculus

<220>
<221> CDS
<222> (1)..(699)
<223> Fc

<400> 19
gag ccc aga ggg ccc aca atc aag ccc tgt cct cca tgc aaa tgc cca      48
Glu Pro Arg Gly Pro Thr Ile Lys Pro Cys Pro Pro Cys Lys Cys Pro
  1               5                  10                  15

gca cct aac ctc ttg ggt gga cca tcc gtc ttc atc ttc cct cca aag      96
Ala Pro Asn Leu Leu Gly Gly Pro Ser Val Phe Ile Phe Pro Pro Lys
             20                  25                  30

atc aag gat gta ctc atg atc tcc ctg agc ccc ata gtc aca tgt gtg     144
Ile Lys Asp Val Leu Met Ile Ser Leu Ser Pro Ile Val Thr Cys Val
         35                  40                  45

gtg gtg gat gtg agc gag gat gac cca gat gtc cag atc agc tgg ttt     192
Val Val Asp Val Ser Glu Asp Asp Pro Asp Val Gln Ile Ser Trp Phe
     50                  55                  60

gtg aac aac gtg gaa gta cac aca gct cag aca caa acc cat aga gag     240
Val Asn Asn Val Glu Val His Thr Ala Gln Thr Gln Thr His Arg Glu
 65                  70                  75                  80

gat tac aac agt act ctc cgg gtg gtc agt gcc ctc ccc atc cag cac     288
Asp Tyr Asn Ser Thr Leu Arg Val Val Ser Ala Leu Pro Ile Gln His
                 85                  90                  95

cag gac tgg atg agt ggc aag gag ttc aaa tgc aag gtc aac aac aaa     336
Gln Asp Trp Met Ser Gly Lys Glu Phe Lys Cys Lys Val Asn Asn Lys
```

```
        100                 105                 110

gac ctc cca gcg ccc atc gag aga acc atc tca aaa ccc aaa ggg tca    384
Asp Leu Pro Ala Pro Ile Glu Arg Thr Ile Ser Lys Pro Lys Gly Ser
        115                 120                 125

gta aga gct cca cag gta tat gtc ttg cct cca cca gaa gaa gag atg    432
Val Arg Ala Pro Gln Val Tyr Val Leu Pro Pro Pro Glu Glu Glu Met
    130                 135                 140

act aag aaa cag gtc act ctg acc tgc atg gtc aca gac ttc atg cct    480
Thr Lys Lys Gln Val Thr Leu Thr Cys Met Val Thr Asp Phe Met Pro
145                 150                 155                 160

gaa gac att tac gtg gag tgg acc aac aac ggg aaa aca gag cta aac    528
Glu Asp Ile Tyr Val Glu Trp Thr Asn Asn Gly Lys Thr Glu Leu Asn
                165                 170                 175

tac aag aac act gaa cca gtc ctg gac tct gat ggt tct tac ttc atg    576
Tyr Lys Asn Thr Glu Pro Val Leu Asp Ser Asp Gly Ser Tyr Phe Met
            180                 185                 190

tac agc aag ctg aga gtg gaa aag aag aac tgg gtg gaa aga aat agc    624
Tyr Ser Lys Leu Arg Val Glu Lys Lys Asn Trp Val Glu Arg Asn Ser
        195                 200                 205

tac tcc tgt tca gtg gtc cac gag ggt ctg cac aat cac cac acg act    672
Tyr Ser Cys Ser Val Val His Glu Gly Leu His Asn His His Thr Thr
    210                 215                 220

aag agc ttc tcc cgg acc ccg ggt aaa                                699
Lys Ser Phe Ser Arg Thr Pro Gly Lys
225                 230


&lt;210&gt; 20
&lt;211&gt; 233
&lt;212&gt; PRT
&lt;213&gt; Mus musculus

&lt;400&gt; 20
Glu Pro Arg Gly Pro Thr Ile Lys Pro Cys Pro Pro Cys Lys Cys Pro
 1               5                  10                  15

Ala Pro Asn Leu Leu Gly Gly Pro Ser Val Phe Ile Phe Pro Pro Lys
            20                  25                  30

Ile Lys Asp Val Leu Met Ile Ser Leu Ser Pro Ile Val Thr Cys Val
        35                  40                  45

Val Val Asp Val Ser Glu Asp Asp Pro Asp Val Gln Ile Ser Trp Phe
    50                  55                  60

Val Asn Asn Val Glu Val His Thr Ala Gln Thr Gln Thr His Arg Glu
65                  70                  75                  80

Asp Tyr Asn Ser Thr Leu Arg Val Val Ser Ala Leu Pro Ile Gln His
                85                  90                  95
```

```
Gln Asp Trp Met Ser Gly Lys Glu Phe Lys Cys Lys Val Asn Asn Lys
            100                 105                 110

Asp Leu Pro Ala Pro Ile Glu Arg Thr Ile Ser Lys Pro Lys Gly Ser
        115                 120                 125

Val Arg Ala Pro Gln Val Tyr Val Leu Pro Pro Pro Glu Glu Glu Met
    130                 135                 140

Thr Lys Lys Gln Val Thr Leu Thr Cys Met Val Thr Asp Phe Met Pro
145                 150                 155                 160

Glu Asp Ile Tyr Val Glu Trp Thr Asn Asn Gly Lys Thr Glu Leu Asn
                165                 170                 175

Tyr Lys Asn Thr Glu Pro Val Leu Asp Ser Asp Gly Ser Tyr Phe Met
            180                 185                 190

Tyr Ser Lys Leu Arg Val Glu Lys Lys Asn Trp Val Glu Arg Asn Ser
        195                 200                 205

Tyr Ser Cys Ser Val Val His Glu Gly Leu His Asn His His Thr Thr
    210                 215                 220

Lys Ser Phe Ser Arg Thr Pro Gly Lys
225                 230


&lt;210&gt; 21
&lt;211&gt; 29
&lt;212&gt; DNA
&lt;213&gt; Artificial Sequence

&lt;220&gt;
&lt;223&gt; Description of Artificial Sequence:Forward primer
      for mouse Fc-Endo

&lt;220&gt;
&lt;221&gt; CDS
&lt;222&gt; (2)..(28)

&lt;400&gt; 21
c ccc aag ctt cat act cat cag gac ttt c                          29
  Pro Lys Leu His Thr His Gln Asp Phe
    1               5


&lt;210&gt; 22
&lt;211&gt; 9
&lt;212&gt; PRT
&lt;213&gt; Artificial Sequence

&lt;400&gt; 22
Pro Lys Leu His Thr His Gln Asp Phe
  1               5


&lt;210&gt; 23
&lt;211&gt; 28
```

<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Reverse primer
for mouse Fc-Endo

<400> 23
cccctcgagc tatttggaga aagaggtc 28

<210> 24
<211> 1086
<212> DNA
<213> Mus musculus

<220>
<221> CDS
<222> (1)..(1086)
<223> Angiostatin

<400> 24
gtg tat ctg tca gaa tgt aag acc ggc atc ggc aac ggc tac aga gga 48
Val Tyr Leu Ser Glu Cys Lys Thr Gly Ile Gly Asn Gly Tyr Arg Gly
1 5 10 15

acc atg tcc agg aca aag agt ggt gtt gcc tgt caa aag tgg ggt gcc 96
Thr Met Ser Arg Thr Lys Ser Gly Val Ala Cys Gln Lys Trp Gly Ala
20 25 30

acg ttc ccc cac gta ccc aac tac tct ccc agt aca cat ccc aat gag 144
Thr Phe Pro His Val Pro Asn Tyr Ser Pro Ser Thr His Pro Asn Glu
35 40 45

gga cta gaa gag aac tac tgt agg aac cca gac aat gat gaa caa ggg 192
Gly Leu Glu Glu Asn Tyr Cys Arg Asn Pro Asp Asn Asp Glu Gln Gly
50 55 60

cct tgg tgc tac act aca gat ccg gac aag aga tat gac tac tgc aac 240
Pro Trp Cys Tyr Thr Thr Asp Pro Asp Lys Arg Tyr Asp Tyr Cys Asn
65 70 75 80

att cct gaa tgt gaa gag gaa tgc atg tac tgc agt gga gaa aag tat 288
Ile Pro Glu Cys Glu Glu Glu Cys Met Tyr Cys Ser Gly Glu Lys Tyr
85 90 95

gag ggc aaa atc tcc aag acc atg tct gga ctt gac tgc cag gcc tgg 336
Glu Gly Lys Ile Ser Lys Thr Met Ser Gly Leu Asp Cys Gln Ala Trp
100 105 110

gat tct cag agc cca cat gct cat gga tac atc cct gcc aaa ttt cca 384
Asp Ser Gln Ser Pro His Ala His Gly Tyr Ile Pro Ala Lys Phe Pro
115 120 125

agc aag aac ctg aag atg aat tat tgc cac aac cct gac ggg gag cca 432
Ser Lys Asn Leu Lys M t Asn Tyr Cys His Asn Pro Asp Gly Glu Pro
130 135 140

agg ccc tgg tgc ttc aca aca gac ccc acc aaa cgc tgg gaa tac tgt 480

```
Arg Pro Trp Cys Phe Thr Thr Asp Pro Thr Lys Arg Trp Glu Tyr Cys
145                 150                 155                 160

gac atc ccc cgc tgc aca aca ccc ccg ccc cca ccc agc cca acc tac     528
Asp Ile Pro Arg Cys Thr Thr Pro Pro Pro Pro Pro Ser Pro Thr Tyr
                165                 170                 175

caa tgt ctg aaa gga aga ggt gaa aat tac cga ggg acc gtg tct gtc     576
Gln Cys Leu Lys Gly Arg Gly Glu Asn Tyr Arg Gly Thr Val Ser Val
            180                 185                 190

acc gtg tct ggg aaa acc tgt cag cgc tgg agt gag caa acc cct cat     624
Thr Val Ser Gly Lys Thr Cys Gln Arg Trp Ser Glu Gln Thr Pro His
        195                 200                 205

agg cac aac agg aca cca gaa aat ttc ccc tgc aaa aat ctg gaa gag     672
Arg His Asn Arg Thr Pro Glu Asn Phe Pro Cys Lys Asn Leu Glu Glu
    210                 215                 220

aac tac tgc cgg aac cca gat gga gaa act gct ccc tgg tgc tat acc     720
Asn Tyr Cys Arg Asn Pro Asp Gly Glu Thr Ala Pro Trp Cys Tyr Thr
225                 230                 235                 240

act gac agc cag ctg agg tgg gag tac tgt gag att cca tcc tgc gag     768
Thr Asp Ser Gln Leu Arg Trp Glu Tyr Cys Glu Ile Pro Ser Cys Glu
                245                 250                 255

tcc tca gca tca cca gac cag tca gat tcc tca gtt cca cca gag gag     816
Ser Ser Ala Ser Pro Asp Gln Ser Asp Ser Ser Val Pro Pro Glu Glu
            260                 265                 270

caa aca cct gtg gtc cag gaa tgc tac cag agc gat ggg cag agc tat     864
Gln Thr Pro Val Val Gln Glu Cys Tyr Gln Ser Asp Gly Gln Ser Tyr
        275                 280                 285

cgg ggt aca tcg tcc act acc atc aca ggg aag aag tgc cag tcc tgg     912
Arg Gly Thr Ser Ser Thr Thr Ile Thr Gly Lys Lys Cys Gln Ser Trp
    290                 295                 300

gca gct atg ttt cca cac agg cat tcg aag acc cca gag aac ttc cca     960
Ala Ala Met Phe Pro His Arg His Ser Lys Thr Pro Glu Asn Phe Pro
305                 310                 315                 320

gat gct ggc ttg gag atg aac tac tgc agg aac ccg gat ggt gac aag     1008
Asp Ala Gly Leu Glu Met Asn Tyr Cys Arg Asn Pro Asp Gly Asp Lys
                325                 330                 335

ggc cct tgg tgc tac acc act gac ccg agc gtc agg tgg gaa tac tgc     1056
Gly Pro Trp Cys Tyr Thr Thr Asp Pro Ser Val Arg Trp Glu Tyr Cys
            340                 345                 350

aac ctg aag cgg tgc tca gag aca gga ggg                             1086
Asn Leu Lys Arg Cys Ser Glu Thr Gly Gly
        355                 360
```

<210> 25
<211> 362
<212> PRT

<213> Mus musculus

<400> 25

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Val Tyr Leu Ser Glu Cys Lys Thr Gly Ile Gly Asn Gly Tyr Arg Gly
  1               5                  10                  15

Thr Met Ser Arg Thr Lys Ser Gly Val Ala Cys Gln Lys Trp Gly Ala
             20                  25                  30

Thr Phe Pro His Val Pro Asn Tyr Ser Pro Ser Thr His Pro Asn Glu
         35                  40                  45

Gly Leu Glu Glu Asn Tyr Cys Arg Asn Pro Asp Asn Asp Glu Gln Gly
     50                  55                  60

Pro Trp Cys Tyr Thr Thr Asp Pro Asp Lys Arg Tyr Asp Tyr Cys Asn
 65                  70                  75                  80

Ile Pro Glu Cys Glu Glu Glu Cys Met Tyr Cys Ser Gly Glu Lys Tyr
                 85                  90                  95

Glu Gly Lys Ile Ser Lys Thr Met Ser Gly Leu Asp Cys Gln Ala Trp
            100                 105                 110

Asp Ser Gln Ser Pro His Ala His Gly Tyr Ile Pro Ala Lys Phe Pro
        115                 120                 125

Ser Lys Asn Leu Lys Met Asn Tyr Cys His Asn Pro Asp Gly Glu Pro
    130                 135                 140

Arg Pro Trp Cys Phe Thr Thr Asp Pro Thr Lys Arg Trp Glu Tyr Cys
145                 150                 155                 160

Asp Ile Pro Arg Cys Thr Thr Pro Pro Pro Pro Pro Ser Pro Thr Tyr
                165                 170                 175

Gln Cys Leu Lys Gly Arg Gly Glu Asn Tyr Arg Gly Thr Val Ser Val
            180                 185                 190

Thr Val Ser Gly Lys Thr Cys Gln Arg Trp Ser Glu Gln Thr Pro His
        195                 200                 205

Arg His Asn Arg Thr Pro Glu Asn Phe Pro Cys Lys Asn Leu Glu Glu
    210                 215                 220

Asn Tyr Cys Arg Asn Pro Asp Gly Glu Thr Ala Pro Trp Cys Tyr Thr
225                 230                 235                 240

Thr Asp Ser Gln Leu Arg Trp Glu Tyr Cys Glu Ile Pro Ser Cys Glu
                245                 250                 255

Ser Ser Ala Ser Pro Asp Gln Ser Asp Ser Ser Val Pro Pro Glu Glu
            260                 265                 270

Gln Thr Pro Val Val Gln Glu Cys Tyr Gln Ser Asp Gly Gln Ser Tyr
        275                 280                 285

Arg Gly Thr Ser Ser Thr Thr Ile Thr Gly Lys Lys Cys Gln Ser Trp
    290                 295                 300
```

Ala Ala Met Phe Pro His Arg His Ser Lys Thr Pro Glu Asn Phe Pro
305 310 315 320

Asp Ala Gly Leu Glu Met Asn Tyr Cys Arg Asn Pro Asp Gly Asp Lys
325 330 335

Gly Pro Trp Cys Tyr Thr Thr Asp Pro Ser Val Arg Trp Glu Tyr Cys
340 345 350

Asn Leu Lys Arg Cys Ser Glu Thr Gly Gly
355 360

<210> 26
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Forward primer
for mouse Fc-Angio

<220>
<221> CDS
<222> (2)..(49)

<400> 26
c ccc aag ctt gtg tat ctg tca gaa tgt aag 31
Pro Lys Leu Val Tyr Leu Ser Glu Cys Lys
1 5 10

<210> 27
<211> 10
<212> PRT
<213> Artificial Sequence

<400> 27
Pro Lys Leu Val Tyr Leu Ser Glu Cys Lys
1 5 10

<210> 28
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Reverse primer
for mouse Fc-Angio

<400> 28
cccctcgagc taccctcctg tctctgagca 30

<210> 29
<211> 29
<212> DNA

<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Forward primer
for canine Fc

<220>
<221> CDS
<222> (3)..(29)

<400> 29
cc tta agc gaa aat gga aga gtt cct cgc 29
Leu Ser Glu Asn Gly Arg Val Pro Arg
1 5

<210> 30
<211> 9
<212> PRT
<213> Artificial Sequence

<400> 30
Leu Ser Glu Asn Gly Arg Val Pro Arg
1 5

<210> 31
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Reverse primer
for canine Fc

<400> 31
cctcgagtca tttacccggg gaatgggaga gggatttctg 40

<210> 32
<211> 702
<212> DNA
<213> Canis familiaris

<220>
<221> CDS
<222> (1)..(702)
<223> Fc

<400> 32
gaa aat gga aga gtt cct cgc cca cct gat tgt ccc aaa tgc cca gcc 48
Glu Asn Gly Arg Val Pro Arg Pro Pro Asp Cys Pro Lys Cys Pro Ala
1 5 10 15

cct gaa atg ctg gga ggg cct tcg gtc ttc atc ttt ccc ccg aaa ccc 96
Pro Glu Met Leu Gly Gly Pro Ser Val Phe Ile Phe Pro Pro Lys Pro
20 25 30

aag gac acc ctc ttg att gcc cga aca cct gag gtc aca tgt gtg gtg 144

Lys Asp Thr Leu Leu Ile Ala Arg Thr Pro Glu Val Thr Cys Val Val
35 40 45

gtg gat ctg gga cca gaa gac cct gag gtg cag atc agc tgg ttc gtg 192
Val Asp Leu Gly Pro Glu Asp Pro Glu Val Gln Ile Ser Trp Phe Val
50 55 60

gac ggt aag cag atg caa aca gcc aag act cag cct cgt gag gag cag 240
Asp Gly Lys Gln Met Gln Thr Ala Lys Thr Gln Pro Arg Glu Glu Gln
65 70 75 80

ttc aat ggc acc tac cgt gtg gtc agt gtc ctc ccc att ggg cac cag 288
Phe Asn Gly Thr Tyr Arg Val Val Ser Val Leu Pro Ile Gly His Gln
85 90 95

gac tgg ctc aag ggg aag cag ttc acg tgc aaa gtc aac aac aaa gcc 336
Asp Trp Leu Lys Gly Lys Gln Phe Thr Cys Lys Val Asn Asn Lys Ala
100 105 110

ctc cca tcc ccg atc gag agg acc atc tcc aag gcc aga ggg cag gcc 384
Leu Pro Ser Pro Ile Glu Arg Thr Ile Ser Lys Ala Arg Gly Gln Ala
115 120 125

cat cag ccc agt gtg tat gtc ctg ccg cca tcc cgg gag gag ttg agc 432
His Gln Pro Ser Val Tyr Val Leu Pro Pro Ser Arg Glu Glu Leu Ser
130 135 140

aag aac aca gtc agc ttg aca tgc ctg atc aaa gac ttc ttc cca cct 480
Lys Asn Thr Val Ser Leu Thr Cys Leu Ile Lys Asp Phe Phe Pro Pro
145 150 155 160

gac att gat gtg gag tgg cag agc aat gga cag cag gag cct gag agc 528
Asp Ile Asp Val Glu Trp Gln Ser Asn Gly Gln Gln Glu Pro Glu Ser
165 170 175

aag tac cgc acg acc ccg ccc cag ctg gac gag gac ggg tcc tac ttc 576
Lys Tyr Arg Thr Thr Pro Pro Gln Leu Asp Glu Asp Gly Ser Tyr Phe
180 185 190

ctg tac agc aag ctc tct gtg gac aag agc cgc tgg cag cgg gga gac 624
Leu Tyr Ser Lys Leu Ser Val Asp Lys Ser Arg Trp Gln Arg Gly Asp
195 200 205

acc ttc ata tgt gcg gtg atg cat gaa gct cta cac aac cac tac aca 672
Thr Phe Ile Cys Ala Val Met His Glu Ala Leu His Asn His Tyr Thr
210 215 220

cag aaa tcc ctc tcc cat tct ccg ggt aaa 702
Gln Lys Ser Leu Ser His Ser Pro Gly Lys
225 230

<210> 33
<211> 234
<212> PRT
<213> Canis familiaris

<400> 33
Glu Asn Gly Arg Val Pro Arg Pro Pro Asp Cys Pro Lys Cys Pro Ala

```
1               5                   10                  15

Pro Glu Met Leu Gly Gly Pro Ser Val Phe Ile Phe Pro Pro Lys Pro
            20                  25                  30

Lys Asp Thr Leu Leu Ile Ala Arg Thr Pro Glu Val Thr Cys Val Val
        35                  40                  45

Val Asp Leu Gly Pro Glu Asp Pro Glu Val Gln Ile Ser Trp Phe Val
    50                  55                  60

Asp Gly Lys Gln Met Gln Thr Ala Lys Thr Gln Pro Arg Glu Glu Gln
65                  70                  75                  80

Phe Asn Gly Thr Tyr Arg Val Val Ser Val Leu Pro Ile Gly His Gln
                85                  90                  95

Asp Trp Leu Lys Gly Lys Gln Phe Thr Cys Lys Val Asn Asn Lys Ala
            100                 105                 110

Leu Pro Ser Pro Ile Glu Arg Thr Ile Ser Lys Ala Arg Gly Gln Ala
        115                 120                 125

His Gln Pro Ser Val Tyr Val Leu Pro Pro Ser Arg Glu Glu Leu Ser
    130                 135                 140

Lys Asn Thr Val Ser Leu Thr Cys Leu Ile Lys Asp Phe Phe Pro Pro
145                 150                 155                 160

Asp Ile Asp Val Glu Trp Gln Ser Asn Gly Gln Gln Glu Pro Glu Ser
                165                 170                 175

Lys Tyr Arg Thr Thr Pro Pro Gln Leu Asp Glu Asp Gly Ser Tyr Phe
            180                 185                 190

Leu Tyr Ser Lys Leu Ser Val Asp Lys Ser Arg Trp Gln Arg Gly Asp
        195                 200                 205

Thr Phe Ile Cys Ala Val Met His Glu Ala Leu His Asn His Tyr Thr
    210                 215                 220

Gln Lys Ser Leu Ser His Ser Pro Gly Lys
225                 230


<210> 34
<211> 552
<212> DNA
<213> Canis familiaris

<220>
<221> CDS
<222> (1)..(552)
<223> Endostatin

<400> 34
cac acc cac cag gac ttc cag ccg gtg ctg cac ctg gtg gcc ctg aac     48
His Thr His Gln Asp Phe Gln Pro Val Leu His Leu Val Ala Leu Asn
1               5                   10                  15
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agc ccg cag ccg ggc ggc atg cga ggc atc cgg gga gcg gac ttc cag 96
Ser Pro Gln Pro Gly Gly Met Arg Gly Ile Arg Gly Ala Asp Phe Gln
20 25 30

tgc ttc cag cag gcg cgc gcc gcg ggg ctg gcc ggc acc ttc cgg gcc 144
Cys Phe Gln Gln Ala Arg Ala Ala Gly Leu Ala Gly Thr Phe Arg Ala
35 40 45

ttc ctg tcg tcg cgg ctg cag gac ctc tac agc atc gtg cgc cgc gcc 192
Phe Leu Ser Ser Arg Leu Gln Asp Leu Tyr Ser Ile Val Arg Arg Ala
50 55 60

gac cgc acc ggg gtg ccc gtc gtc aac ctc agg gac gag gtg ctc ttc 240
Asp Arg Thr Gly Val Pro Val Val Asn Leu Arg Asp Glu Val Leu Phe
65 70 75 80

ccc agc tgg gag gcc tta ttc tcg ggc tcc gag ggc cag ctg aag ccc 288
Pro Ser Trp Glu Ala Leu Phe Ser Gly Ser Glu Gly Gln Leu Lys Pro
85 90 95

ggg gcc cgc atc ttc tct ttc gac ggc aga gat gtc ctg cag cac ccc 336
Gly Ala Arg Ile Phe Ser Phe Asp Gly Arg Asp Val Leu Gln His Pro
100 105 110

gcc tgg ccc cgg aag agc gtg tgg cac ggc tcc gac ccc agc ggg cgc 384
Ala Trp Pro Arg Lys Ser Val Trp His Gly Ser Asp Pro Ser Gly Arg
115 120 125

cgc ctg acc gac agc tac tgc gag acg tgg cgg acg gag gcc ccg gcg 432
Arg Leu Thr Asp Ser Tyr Cys Glu Thr Trp Arg Thr Glu Ala Pro Ala
130 135 140

gcc acc ggg cag gcg tcg tcg ctg ctg gcg ggc agg ctg ctg gag cag 480
Ala Thr Gly Gln Ala Ser Ser Leu Leu Ala Gly Arg Leu Leu Glu Gln
145 150 155 160

gag gcc gcg agc tgc cgc cac gcc ttc gtg gtg ctc tgc atc gag aac 528
Glu Ala Ala Ser Cys Arg His Ala Phe Val Val Leu Cys Ile Glu Asn
165 170 175

agc gtc atg acc tcc ttc tcc aag 552
Ser Val Met Thr Ser Phe Ser Lys
180

<210> 35
<211> 184
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<213> Canis familiaris

<400> 35
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Ser Pro Gln Pro Gly Gly Met Arg Gly Ile Arg Gly Ala Asp Phe Gln
20 25 30

Cys Phe Gln Gln Ala Arg Ala Ala Gly Leu Ala Gly Thr Phe Arg Ala

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        35                  40                  45

Phe Leu Ser Ser Arg Leu Gln Asp Leu Tyr Ser Ile Val Arg Arg Ala
     50                  55                  60

Asp Arg Thr Gly Val Pro Val Val Asn Leu Arg Asp Glu Val Leu Phe
 65                  70                  75                  80

Pro Ser Trp Glu Ala Leu Phe Ser Gly Ser Glu Gly Gln Leu Lys Pro
                 85                  90                  95

Gly Ala Arg Ile Phe Ser Phe Asp Gly Arg Asp Val Leu Gln His Pro
            100                 105                 110

Ala Trp Pro Arg Lys Ser Val Trp His Gly Ser Asp Pro Ser Gly Arg
        115                 120                 125

Arg Leu Thr Asp Ser Tyr Cys Glu Thr Trp Arg Thr Glu Ala Pro Ala
    130                 135                 140

Ala Thr Gly Gln Ala Ser Ser Leu Leu Ala Gly Arg Leu Leu Glu Gln
145                 150                 155                 160

Glu Ala Ala Ser Cys Arg His Ala Phe Val Val Leu Cys Ile Glu Asn
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      linker: top strand

<220>
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<222> (3)..(41)

<400> 36
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   Leu His Thr His Gln Asp Phe Gln Pro Val Leu His Leu
     1               5                  10


<210> 37
<211> 13
<212> PRT
<213> Artificial Sequence

<400> 37
Leu His Thr His Gln Asp Phe Gln Pro Val Leu His Leu
  1               5                  10
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<210> 38
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
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linker: bottom strand

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<210> 39
<211> 1077
<212> DNA
<213> Canis familiaris

<220>
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<222> (1)..(1077)
<223> angiostatin

<400> 39
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Ile Tyr Leu Ser Glu Cys Lys Thr Gly Asn Gly Lys Thr Tyr Arg Gly
1 5 10 15

acc atg gcc aaa acg aag aat gat gtt gcc tgt caa aaa tgg agt gac 96
Thr Met Ala Lys Thr Lys Asn Asp Val Ala Cys Gln Lys Trp Ser Asp
20 25 30

aat tct ccg cac aaa cct aac tat acg cct gag aag cac ccc ttg gag 144
Asn Ser Pro His Lys Pro Asn Tyr Thr Pro Glu Lys His Pro Leu Glu
35 40 45

ggg ctg gag gag aac tat tgc agg aac cct gac aac gac gag aac ggg 192
Gly Leu Glu Glu Asn Tyr Cys Arg Asn Pro Asp Asn Asp Glu Asn Gly
50 55 60

ccc tgg tgc tac acc aca aac cca gac gtg agg ttc gac tac tgc aac 240
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65 70 75 80

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Ile Pro Glu Cys Glu Glu Glu Cys Met His Cys Ser Gly Glu Asn Tyr
85 90 95

gag ggc aaa att tcc aag aca aag tct gga ctc gag tgc caa gcc tgg 336
Glu Gly Lys Ile Ser Lys Thr Lys Ser Gly Leu Glu Cys Gln Ala Trp
100 105 110

aac tct caa acc cca cat gct cat gga tat att cct tcc aaa ttt cca 384
Asn Ser Gln Thr Pro His Ala His Gly Tyr Ile Pro Ser Lys Phe Pro
115 120 125

agc aag aac ttg aag atg aat tac tgc cgt aac cct gat ggg gag ccc 432
Ser Lys Asn Leu Lys Met Asn Tyr Cys Arg Asn Pro Asp Gly Glu Pro
130 135 140

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cgc cca tgg tgt ttc acc atg gat ccc aac aaa cgc tgg gaa ttc tgt      480
Arg Pro Trp Cys Phe Thr Met Asp Pro Asn Lys Arg Trp Glu Phe Cys
145                 150                 155                 160

gac att ccc cgc tgt aca aca cca cca ccc cct tcg ggc cca acg tac      528
Asp Ile Pro Arg Cys Thr Thr Pro Pro Pro Pro Ser Gly Pro Thr Tyr
                165                 170                 175

cag tgt ctg aag ggc aga ggg gag agc tac cga ggg aag gtg tcc gtc      576
Gln Cys Leu Lys Gly Arg Gly Glu Ser Tyr Arg Gly Lys Val Ser Val
            180                 185                 190

act gtc tct gga cat aca tgt cag cac tgg agt gaa cag acc cct cac      624
Thr Val Ser Gly His Thr Cys Gln His Trp Ser Glu Gln Thr Pro His
        195                 200                 205

aag cac aac agg acc cca gaa aac ttc cct tgc aaa aat ttg gat gaa      672
Lys His Asn Arg Thr Pro Glu Asn Phe Pro Cys Lys Asn Leu Asp Glu
    210                 215                 220

aac tac tgt cgc aac cct gat gga gaa aca gct cca tgg tgc tac aca      720
Asn Tyr Cys Arg Asn Pro Asp Gly Glu Thr Ala Pro Trp Cys Tyr Thr
225                 230                 235                 240

acc aac agt gag gtg agg tgg gaa cac tgc cag att ccg tcc tgt gag      768
Thr Asn Ser Glu Val Arg Trp Glu His Cys Gln Ile Pro Ser Cys Glu
                245                 250                 255

tcc tct cca ata acc aca gaa tat ttg gat gcc cca gct tca gtg cca      816
Ser Ser Pro Ile Thr Thr Glu Tyr Leu Asp Ala Pro Ala Ser Val Pro
            260                 265                 270

cct gaa caa act cct gtg gtc cag gag tgc tac cac ggc aat ggg cag      864
Pro Glu Gln Thr Pro Val Val Gln Glu Cys Tyr His Gly Asn Gly Gln
        275                 280                 285

agt tat cga ggc aca tca tcc act act atc aca gga aga aaa tgt cag      912
Ser Tyr Arg Gly Thr Ser Ser Thr Thr Ile Thr Gly Arg Lys Cys Gln
    290                 295                 300

tct tgg tca tct atg aca cca cac cga cat gag aag acc cca gaa cac      960
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305                 310                 315                 320

ttc ccg gag gct ggc ctg aca atg aac tac tgc agg aat ccc gac gcc      1008
Phe Pro Glu Ala Gly Leu Thr Met Asn Tyr Cys Arg Asn Pro Asp Ala
                325                 330                 335

gac aaa agc cct tgg tgt tac acc acc gac ccc tct gtg cgc tgg gag      1056
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<213> Canis familiaris

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Thr Met Ala Lys Thr Lys Asn Asp Val Ala Cys Gln Lys Trp Ser Asp
            20                  25                  30

Asn Ser Pro His Lys Pro Asn Tyr Thr Pro Glu Lys His Pro Leu Glu
        35                  40                  45

Gly Leu Glu Glu Asn Tyr Cys Arg Asn Pro Asp Asn Asp Glu Asn Gly
    50                  55                  60

Pro Trp Cys Tyr Thr Thr Asn Pro Asp Val Arg Phe Asp Tyr Cys Asn
65                  70                  75                  80

Ile Pro Glu Cys Glu Glu Glu Cys Met His Cys Ser Gly Glu Asn Tyr
                85                  90                  95

Glu Gly Lys Ile Ser Lys Thr Lys Ser Gly Leu Glu Cys Gln Ala Trp
            100                 105                 110

Asn Ser Gln Thr Pro His Ala His Gly Tyr Ile Pro Ser Lys Phe Pro
        115                 120                 125

Ser Lys Asn Leu Lys Met Asn Tyr Cys Arg Asn Pro Asp Gly Glu Pro
    130                 135                 140

Arg Pro Trp Cys Phe Thr Met Asp Pro Asn Lys Arg Trp Glu Phe Cys
145                 150                 155                 160

Asp Ile Pro Arg Cys Thr Thr Pro Pro Pro Pro Ser Gly Pro Thr Tyr
                165                 170                 175

Gln Cys Leu Lys Gly Arg Gly Glu Ser Tyr Arg Gly Lys Val Ser Val
            180                 185                 190

Thr Val Ser Gly His Thr Cys Gln His Trp Ser Glu Gln Thr Pro His
        195                 200                 205

Lys His Asn Arg Thr Pro Glu Asn Phe Pro Cys Lys Asn Leu Asp Glu
    210                 215                 220

Asn Tyr Cys Arg Asn Pro Asp Gly Glu Thr Ala Pro Trp Cys Tyr Thr
225                 230                 235                 240

Thr Asn Ser Glu Val Arg Trp Glu His Cys Gln Ile Pro Ser Cys Glu
                245                 250                 255

Ser Ser Pro Ile Thr Thr Glu Tyr Leu Asp Ala Pro Ala Ser Val Pro
            260                 265                 270

Pro Glu Gln Thr Pro Val Val Gln Glu Cys Tyr His Gly Asn Gly Gln
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Ser Tyr Arg Gly Thr Ser Ser Thr Thr Ile Thr Gly Arg Lys Cys Gln
    290                 295                 300

Ser Trp Ser Ser Met Thr Pro His Arg His Glu Lys Thr Pro Glu His
305                 310                 315                 320

Phe Pro Glu Ala Gly Leu Thr Met Asn Tyr Cys Arg Asn Pro Asp Ala
                325                 330                 335

Asp Lys Ser Pro Trp Cys Tyr Thr Thr Asp Pro Ser Val Arg Trp Glu
            340                 345                 350

Phe Cys Asn Leu Arg Lys Cys
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<210> 41
<211> 12
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<213> Artificial Sequence

<220>
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<400> 41
tgactcgagt ca                                                        12


<210> 42
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
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      primer for murine angiostatin

<220>
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<222> (1)..(21)

<400> 42
ggg cct tgg agc tac act aca                                          21
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<210> 43
<211> 7
<212> PRT
<213> Artificial Sequence

<400> 43
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<210> 44
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
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      introduce HindIII into murine angiostatin

<220>
<221> CDS
<222> (9)..(32)

<400> 44
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         Lys Leu Ser Thr His Pro Asn Glu
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<210> 45
<211> 8
<212> PRT
<213> Artificial Sequence

<400> 45
Lys Leu Ser Thr His Pro Asn Glu
  1               5


<210> 46
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<220>
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<222> (2)..(58)

<400> 46
c atg acc tct ttc tcc aaa tcg agc ggg ggc agc ggg ggc gga ggc agc 49
  Met Thr Ser Phe Ser Lys Ser Ser Gly Gly Ser Gly Gly Gly Gly Ser
    1               5                  10                  15

ggc ggg ggc g                                                     59
Gly Gly Gly


<210> 47
<211> 19
<212> PRT
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<400> 47
Met Thr Ser Phe Ser Lys Ser Ser Gly Gly Ser Gly Gly Gly Gly Ser
  1               5                  10                  15
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<210> 48
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<212> DNA
<213> Artificial Sequence

<220>
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<400> 48
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<213> Artificial Sequence

<220>
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<400> 49
ga tcc tca ggc c 12
Ser Ser Gly
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<210> 50
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<400> 50
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<210> 51
<211> 10
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<213> Artificial Sequence

<220>
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linker: top strand

<220>
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<400> 51
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Leu Ser Gly
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<210> 52
<211> 10
<212> DNA
<213> Artificial Sequence

<220>
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<400> 52
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<210> 53
<211> 11
<212> DNA
<213> Artificial Sequence

<220>
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linker: top strand

<220>
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<222> (3)..(11)

<400> 53
tc gac tcc ggc 11
Asp Ser Gly
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<210> 54
<211> 11
<212> DNA
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<220>
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linker: bottom strand

<400> 54
ttaagccgga g 11

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Fig. 1A

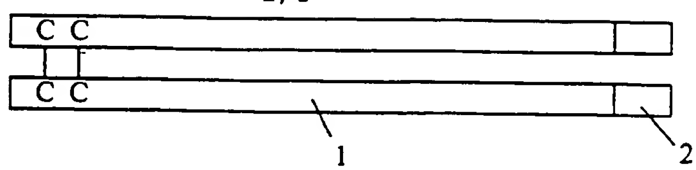


Fig. 1B

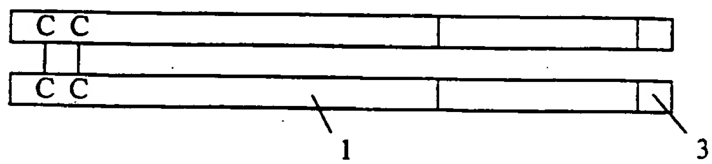


Fig. 1C

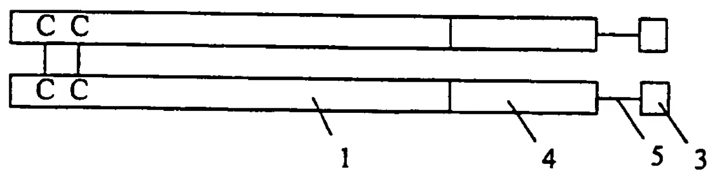


Fig. 1D

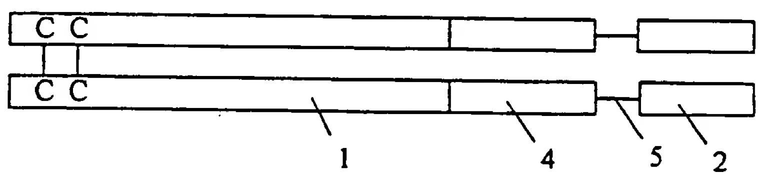


Fig. 1E

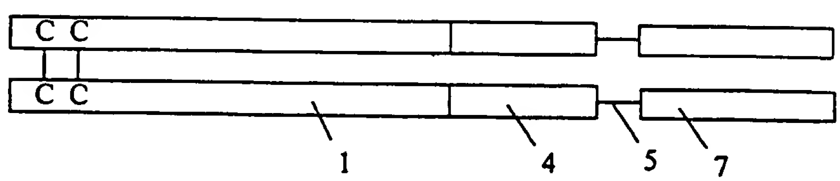
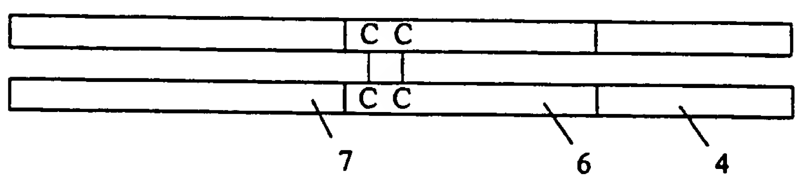


Fig. 1F



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(71) Applicant: LEXINGEN PHARMACEUTICALS . CORP. [US/US]; 125 Hartwell Avenue, Lexington, MA 02173 (US).

(72) Inventors: LO, Kin-Ming; 6 Carol Lane, Lexington, MA 02420 (US). LI, Yue; 53 Loomis Street, Bedford, MA 01730 (US). GILLIES, Stephen, D.; 159 Sunset Road, Carlisle, MA 01741 (US).

(74) Agent: BRESNAHAN, Maureen, B.; Testa, Hurwitz & Thibeault, LLP, High Street Tower, 125 High Street, Boston, MA 02110 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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(54) Title: EXPRESSION AND EXPORT OF ANGIOSTATIN AND ENDOSTATIN AS IMMUNOFUSINS

(57) Abstract

Disclosed are nucleotide sequences, for example, DNA or RNA sequences, which encode an immunoglobulin Fc-angiogenesis inhibitor fusion protein. The angiogenesis inhibitors can be angiostatin, endostatin, a plasminogen fragment having angiostatin activity, or a collagen XVIII fragment having endostatin activity. The nucleotide sequences can be inserted into a suitable expression vector and expressed in mammalian cells. Also disclosed is a family of immunoglobulin Fc-angiogenesis inhibitor fusion proteins that can be produced by expression of such nucleotide sequences. Also disclosed are methods using such nucleotide sequences and fusion proteins for treating conditions mediated by angiogenesis.

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INTERNATIONAL SEARCH REPORT

Intl... ional Application No
PCT/US 99/19329

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/78 C07K14/515 C12N9/68 C12N15/62 C07K19/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SIM ET AL: "A recombinant human angiostatin protein inhibits experimental primary and metastatic cancer" CANCER RESEARCH,US,AMERICAN ASSOCIATION FOR CANCER RESEARCH, vol. 57, no. 7, 1 April 1997 (1997-04-01), page 1329-1334 XP002100107 ISSN: 0008-5472 the whole document ---	1-12, 15-17, 22-24, 26-31
Y	O'REILLY M S ET AL: "Endostatin: an endogenous inhibitor of angiogenesis and tumor growth" CELL,US,CELL PRESS, CAMBRIDGE, NA, vol. 88, 24 January 1997 (1997-01-24), pages 277-285, XP002100111 ISSN: 0092-8674 the whole document ---	1-11, 13-17, 22-24, 26-31

-/--

[X] Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search	Date of mailing of the international search report
21 March 2000	03.04.00

Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	LO K-M ET AL.: "High level expression and secretion of Fc-X fusion proteins in mammalian cells" PROTEIN ENGINEERING, vol. 11, no. 6, June 1998 (1998-06), pages 495-500, XP002125745 cited in the application the whole document ---	1-17, 22-24, 26-31
Y	BACHELOT ET AL: "Retrovirus-mediated gene transfer of an angiostatin-endostatin fusion protein with enahnced anti-tumor properties in vivo" PROCEEDINGS OF THE ANNUAL MEETING OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, vol. 39, March 1998 (1998-03), page 271 XP002089298 see abstract number 1856 the whole document ---	1,11,15, 23
A	WU Z ET AL: "Suppression of tumor growth with recombinant murine angiostatin" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS,US,ACADEMIC PRESS INC., vol. 236, no. 3, 30 July 1997 (1997-07-30), page 651-654 XP002113046 ISSN: 0006-291X the whole document ---	
A	WO 96 08570 A (FUJI IMMUNOPHARMACEUTICALS COR) 21 March 1996 (1996-03-21) cited in the application the whole document ---	
A	E HOHENESTER ET AL: "Crystal structure of the angiogenesis inhibitor endostatin at 1.5 A resolution" EMBO JOURNAL,GB,OXFORD UNIVERSITY PRESS, SURREY, vol. 17, no. 6, 1998, pages 1656-1664-1664, XP002100418 ISSN: 0261-4189 the whole document ---	
P,X	Y-H DING ET AL: "Zinc-dependent dimers observed in crystals of human endostatin" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 95, no. 18, September 1998 (1998-09), pages 10443-10448-10448, XP002100420 ISSN: 0027-8424 the whole document --- -/--	1-11,13, 14,16, 17,26-31

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/US 99/19329

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
E	WO 99 62944 A (CHILDRENS MEDICAL CENTER) 9 December 1999 (1999-12-09) the whole document -----	1-11, 13-18, 21-31

3

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT	International application No. PCT/US 99/ 19329

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 29 - 31 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See additional sheet

1. [X] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[X] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-11, 15-19, 22-31 all partially; 12, 20 all complete

   A DNA molecule encoding a fusion protein comprising a signal sequence, an immunoglobulin Fc region and a target sequence which is selected from angiostatin or a plasminogen fragment having angiostatin activity. A fusion protein, a multimeric protein encoded by said DNA molecule. A vector, a cell comprising said DNA.

2. Claims: 1-11, 15-19, 22-31 all partially; 13, 14, 21 all complete

   same as invention 1 but wherein the target sequence is selected from endostatin and a collagen XVIII fragment having endostatin activity.

INTERNATIONAL SEARCH REPORT

Information on patent family members

In dional Application No
PCT/US 99/19329

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9608570 A	21-03-1996	US 5541087 A	30-07-1996
		AU 691980 B	28-05-1998
		AU 3676595 A	29-03-1996
		CA 2199830 A	21-03-1996
		EP 0782625 A	09-07-1997
		JP 2877959 B	05-04-1999
		JP 10505751 T	09-06-1998
		US 5726044 A	10-03-1998
WO 9962944 A	09-12-1999	NONE	